VOL. 39 MARCH, 1952 No. 3

UNIVERSITY OF MICHIGAN
MAR 28 1952
MEDICAL LIBRARY

The Journal of Laboratory and Clinical Medicine

The Official Publication of the Central Society for Clinical Research

Clinical and Experimental

Metabolism of Iron. I. Intravenous Iron Tolerance Tests in Normal Subjects and Patients With Hemochromatosis. Stanley E. Gitlow, M.D., and Milton R. Beyers, M.D., New York, N. Y. ---- 337

The Blood Volume in Portal Cirrhosis as Determind by P^{32} Labeled Red Blood Cells. Grace M. Hyde, M.D., Nathaniel I. Berlin, M.D., Robert J. Parsons, M.D., John H. Lawrence, M.D., and Shirley Port, B.A., Berkeley, Calif., and Oakland, Calif. ---- 347

Studies on the Separation of Cell Types in Serosanguinous Fluids, Blood, and Vaginal Fluids by Flotation on Bovine Plasma Albumin. Don W. Fawcett, M.D., and Bert L. Vallee, M.D., Boston, Mass. ---- 354

The Response of Monocytes to Adrenal Cortical Extract. Edna H. Tompkins, M.D., Boston, Mass. ---- 365

Response of the Serum Polysaccharide Fractions and Protein Fractions Following Cortisone Treatment of Patients With Rheumatic Fever. M. R. Shetlar, Ph.D., Henry L. Schmidt, Jr., M.D., Richard B. Lincoln, M.D., James K. DeVore, M.D., Jane A. Bullock, M.S., and Arthur A. Hellbaum, M.D., Ph.D., Oklahoma City, Okla. ---- 372

Studies of the Cortisone Effects on the Inflammatory Response. I. Alterations of the Histopathology of Chemically Induced Inflammation. David M. Spain, M.D., Norman Molomut, Ph.D., and Alvin Haber, B.S., Valhalla, N. Y., and Brooklyn, N. Y. ---- 383

Plasma and Red Cell Catalase in Rheumatoid Arthritis. M. R. Jeffrey, M.B., M.R.C.P., Bath, England ---- 390

Electrolyte Changes Produced by ACTH, Cortisone, and DOCA in Cirrhosis of the Liver. William H. Blahd, M.D., William S. Adams, M.D., Alan Leslie, M.D., Ralph Goldman, M.D., and Samuel H. Bassett, M.D., Los Angeles, Calif. ---- 393

The Estimation of Insulin Sensitivity by the Modified Glucose Insulin Tolerance Test. Sydney S. Lazarus, M.D., and Bruno W. Volk, M.D., Brooklyn, N. Y. ---- 404

The Disappearance of Intravenously Injected Emulsified Fat From the Circulation of Patients and Animals. William A. Johnson, M.D., Smith Freeman, M.D., Ph.D., and Karl A. Meyer, M.D., Chicago, Ill. ---- 414

The Effect of Sweating on Normo- and Hypertensive Subjects. Thomas B. Gibbons, M.D., and Carleton B. Chapman, M.D., Minneapolis, Minn. ---- 420

Results in the Hemagglutination Test for Tuberculosis. William Hentel, M.D., and G. D. Guilbert, M.D., Kerrville, Texas ---- 426

(Contents continued on inside front cover)

Published by THE C. V. MOSBY COMPANY, ST. LOUIS 3, MISSOURI

Copyright 1952 by The C. V. Mosby Company

Contents—(Continued from front cover)

The Early Development of Anatomic and Blood Chemistry Changes in the Nephrotic Syndrome in Rats. Walter Heymann, M.D., and Donald B. Hackel, M.D., Cleveland, Ohio ---- 429

Studies on the Technique of Transporting Throat Swabs to the Laboratory for the Isolation of Pneumococci. David A. Stevens, A.B., Cleveland, Ohio ---- 437

Cold Hemagglutinins: Effect of Storage at −20°C. A. E. Feller, M.D., Cleveland, Ohio ---- 440

Hemolysis of Red Blood Cells Treated by Bacterial Filtrates in the Presence of Serum and Complement. Lowell A. Rantz, M.D., Ann Zuckerman, A.B., and Elizabeth Randall, M.A., San Francisco, Calif. ---- 443

Further Studies on the Nature of the Hemorrhagic State in Radiation Injury. D. P. Jackson, M.D., E. P. Cronkite, M.D., G. V. Leroy, M.D., and Bernard Halpern, M.S., Bethesda, Md. ---- 449

The Treatment of Postirradiation Infection With Antibiotics; an Experimental Study on Mice. C. Phillip Miller, M.D., Carolyn W. Hammond, Ph.D., Marianne Tompkins, B.S., and Gertrude Shorter, Chicago, Ill. ---- 462

Laboratory Methods

Simple Method for Demonstrating the "L.E." Phenomenon in Peripheral Blood. Austin S. Weisberger, M.D., Gordon C. Meacham, M.D., and Robert W. Heinle, M.D., Cleveland, Ohio ---- 480

Sheep Blood Agar for the Isolation of Lancefield Groups of Beta-Hemolytic Streptococci. A. E. Feller, M.D., and David A. Stevens, A.B., Cleveland, Ohio ---- 484

A Mechanical Sampling Device for Use With the Standard Electrophoresis Apparatus. Demetrios A. Rigas, Ph.D., Thomas J. Maupin, and Carl G. Heller, M.D., Ph.D., Portland, Ore. ---- 492

A Comparison of Saponin and Aerosol as Hemolytic Agents in the Determination of Blood Gases. Steven M. Horvath, Ph.D., and Doris W. Knapp, M.S., Iowa City, Iowa ---- 495

Spectrophotometric Analysis of Carbohydrates and Study of Anthrone Reagent. R. E. Zipf, M.D., and A. L. Waldo, M.S., Dayton, Ohio ---- 497

Mucin Solution for Enhancing the Virulence of S. Typhi. Lucy Mishulow, B.A., New York, N. Y. ---- 503

Editor
CLAYTON G. LOOSLI, M.D.

Associate Editor
MATTHEW BLOCK, M.D.

University of Chicago School of Medicine
950 East 59th Street
Chicago 37, Illinois

Board of Editors

S. HOWARD ARMSTRONG, Jr., M.D.
Presbyterian Hospital, Chicago

WILLIAM B. BEAN, M.D.
State University of Iowa, Iowa City

KENNETH M. BRINKHOUS, M.D.
University of North Carolina
Chapel Hill

A. C. CORCORAN, M.D.
Cleveland Clinic, Cleveland

CHARLES A. DOAN, M.D.
Ohio State University, Columbus

EUGENE B. FERRIS, Jr., M.D.
Cincinnati General Hospital, Cincinnati

E. M. K. GEILING, M.D.
University of Chicago, Chicago

THOMAS H. HUNTER, M.D.
Washington University, St. Louis

LEON O. JACOBSON, M.D.
University of Chicago, Chicago

CARL V. MOORE, M.D.
Washington University, St. Louis

WESLEY W. SPINK, M.D.
University of Minnesota, Minneapolis

RANDALL G. SPRAGUE, M.D.
Mayo Clinic, Rochester

CECIL J. WATSON, M.D.
University of Minnesota, Minneapolis

Vol. 39, No. 3, March, 1952. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription Price: United States, its possessions, Pan-American Countries, $10.00 per year; Canada, $11.00; Foreign, $11.00. Subscriptions to medical students, interns and residents, half price. Entered as Second-Class Matter, October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

METABOLISM OF IRON

I. Intravenous Iron Tolerance Tests in Normal Subjects and Patients with Hemochromatosis

Stanley E. Gitlow, M.D.,* and Milton R. Beyers, M.D.**
New York, N. Y.

REPEATED phlebotomies for the removal of excess storage iron in hemochromatosis have been used empirically for many years. It has been recently proved that these excess iron stores are available for hemoglobin synthesis.[1] This established the rationale for such therapy.[2, 3]

Unfortunately, the majority of patients with hemachromatosis are diagnosed at both an advanced age and a relatively advanced stage of the disease at which time they may be unable to withstand the rigors of this regimen. Early diagnosis is therefore imperative. The clinical diagnosis of this disease is often difficult or impossible without laboratory aids. Liver biopsy, skin biopsy, and the finding of hemosiderin in the bone marrow have been, until recently, the diagnostic aids of choice. Of these only the liver biopsy is diagnostic in every case of hemochromatosis. Since liver biopsy is not an innocuous procedure, some other technique would be desirable for screening suspected cases of hemochromatosis.

A high serum iron with a high degree of saturation of the total iron-binding capacity has been noted in hemochromatosis.[4-8] However, normal serum iron values and even hypoferremia have been reported in this disease.[9-12]

It was felt that intravenous iron tolerance tests[6, 13, 14] might not only aid in diagnosis, but also shed some light on the metabolism of iron in hemochromatosis. The test was performed in fasting subjects by the slow intravenous administration of saline-diluted green ferric ammonium citrate, in an amount equivalent to ten milligrams of elemental iron. Serum iron determinations were performed[15] on specimens drawn prior to, five minutes after, and one hundred twenty minutes after the injection of the iron. It has been conclusively demonstrated that the ten milligrams of iron injected is in excess of that quantity necessary to saturate the serum iron-binding protein completely.[5, 14, 16, 17, 18] The iron in excess of that amount bound by the iron-binding protein diffuses rapidly into the tissues, giving rise to the toxic symptoms of sneezing, coughing,

Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own studies and do not necessarily reflect the opinion or policy of the Veterans Administration.

From the Department of Internal Medicine, Veterans Administration Hospital, Kingsbridge Road, Bronx, N. Y.

Read in part before the Eastern Section of the American Federation for Clinical Research, Washington, D. C., Dec. 9, 1950.

Received for publication, Aug. 6, 1951.

*Research Fellow, Department of Pharmacology, University of the State of New York, College of Medicine, Brooklyn, New York.

**Resident in Medicine, Veterans Administration Hospital, Kingsbridge Road, Bronx, N. Y.

flushing, nausea, and occasionally vomiting. The injection of iron increases the serum iron from the fasting level (SeFe) to the total iron-binding capacity of the serum (TIBC). The latter is represented by the five-minute specimen.[16] This maximal serum iron level has also been called the braking point. The increase (INC) from the fasting serum iron level (SeFe) to the total iron-binding capacity level (TIBC) is the unsaturated iron-binding capacity. The decrease (DEC) in the serum iron level from the five-minute to the one-hundred-twenty-minute specimen is apparently a function of the tissue uptake of the serum iron.

METHODS

Intravenous iron tolerance tests were performed on ten normal males, ranging from twenty to forty years in age. None of these controls revealed any evidence of infection, blood loss, or neoplasm in recent history or physical and laboratory examinations. Thirteen intravenous iron tolerance tests were performed on seven patients with hemachromatosis proved by liver biopsy. All of the patients except one (P. F.) had manifest diabetes well controlled by diet and insulin therapy. In six of the seven, there was no evidence of fever, acute infection, ascites, edema, neoplasia, or anemia. One patient (J. C.), to be discussed in detail later, had a cholangiocarcinoma and a normochromic anemia. As judged by clinical observation and laboratory findings, no case revealed any evidence of active hepatitis.

The serum iron determinations were performed by a minor modification of the technique described by Balle.[15] An accuracy of ±5 per cent was attained with this method. Recovery studies were within 95 to 105 per cent. The intravenous iron solution was prepared by diluting 1.3 milliliters of the intramuscular preparation of green ferric ammonium citrate (1 milliliter equivalent to 0.05 gram of the iron salt) with 8.7 milliliters of sterile saline. This 10-milliliter volume was administered intravenously over a five-minute period. The first blood specimen (SeFe) was withdrawn immediately prior to this injection, and the second and third specimens were taken five and one-hundred twenty minutes after the completion of the injection. All syringes and other glassware were washed with hydrochloric acid and iron-free water. All iron determinations were done in triplicate.

RESULTS

Results are presented in Tables I and II.

TABLE I. INTRAVENOUS IRON TOLERANCE TESTS IN NORMAL MALES

	SeFe (γ%)	TIBC (γ%)	120′ (γ%)	SATURATION (%)	INC (γ%)	DEC (γ%)	DEC/INC RATIO
A.	74	253	163	29	179	90	0.50
B.	109	309	226	28	200	83	0.41
C.	145	346	326	42	201	20	0.10
D.	136	335	303	41	199	32	0.16
E.	180	308	289	58	128	19	0.15
F.	186	364	333	51	178	31	0.17
G.	177	371	335	48	194	36	0.19
H.	139	388	328	36	249	60	0.24
I.	139	388	321	36	249	67	0.27
J.	153	344	263	44	191	81	0.42
Average values	146	341	289	41	196	52	0.26

SeFe = fasting serum iron; TIBC = total iron binding capacity (5′ specimen); saturation = SeFe/TIBC × 100; INC = increase (TIBC—SeFe); DEC = decrease (TIBC—120′).

Normals.—The SeFe values ranged from 74 μg to 186 μg per 100 ml. of serum, with a mean of 146 μg per cent. The TIBC values ranged from 253 to 388 μg per cent, and averaged 341 μg per cent. The average saturation of the

TABLE II. INTRAVENOUS IRON TOLERANCE TESTS IN PATIENTS WITH HEMOCHROMATOSIS

	SeFe (γ%)	TIBC (γ%)	120′ (γ%)	SATURATION (%)	INC (γ%)	DEC (γ%)	DEC/INC RATIO
P. F.							
8/21/50	257	294	272	87	37	22	0.59
10/27/50	297	326	326	91	29	0	---
W. L.							
9/12/50	196	222	124	88	26	98	3.77
9/14/50	133	212	114	63	79	98	1.24
11/16/50	201	222	167	91	21	55	2.62
J. Mu.							
10/2/50	286	304	260	94	18	36	2.00
10/9/50	290	301	255	96	11	46	4.18
J. Mo.							
10/20/50	204	207	199	99	3	16	5.33
11/8/50	197	207	187	95	10	20	2.00
*T. C.**							
4/1/51	273	335	281	82	62	54	0.87
A. E.							
5/17/51	153	195	164	78	42	31	0.74
5/26/51	120	164	131	73	44	33	0.75
J. C.							
5/19/51	168	206	171	82	38	35	0.92
Average values	213	246	204	94	32	42	2.09

SeFe = fasting serum iron; TIBC = total iron binding capacity (5′ specimen); saturation = SeFe/TIBC × 100; INC = increase (TIBC—SeFe); DEC = decrease (TIBC—120′).

*This patient was studied through the courtesy of Dr. L. Leiter, Montefiore Hospital, New York.

serum iron-binding protein was 41 per cent, with a range of 28 to 58 per cent. The serum iron concentrations decreased from the peak TIBC values to an average of 289 μg per cent at one hundred twenty minutes. The latter ranged from 163 to 335 μg per cent. Although the absolute values for the serum iron increase (INC=TIBC—SeFe) and decrease (DEC=TIBC—one-hundred-twenty-minute specimen) varied, the ratio, DEC/INC, was observed to be fairly constant. It varied from 0.10 to 0.50, averaging 0.26. The significance of this ratio will be discussed later.

Hemochromatosis.—The SeFe values varied from 120 to 297 μg per cent, with an average of 213 μg per cent. The TIBC averaged 246 μg per cent, with a range of 164 to 335 μg per cent. The average saturation of the serum iron-binding capacity was 94 per cent, varying from 63 to 99 per cent. At one hundred twenty minutes the serum iron values ranged from 114 to 326 μg per cent, with a mean of 204 μg per cent. The ratio, DEC/INC, varied from 0.59 to 5.33, averaging 2.09. It should be noted that the majority of the calculated ratios were greater than 1.0 and that only one was less than 0.74. A repeat testing in the patient with the ratio of 0.59 (P. F.) revealed an INC of 29 μg per cent and a DEC of zero, with a resultant incalculable DEC/INC. The possible significance of this will be mentioned below.

DISCUSSION

Although the average fasting serum iron level in our normal patients was slightly higher than those average levels noted in Table III, it was in fair agreement with the determinations done previously in which a similar chemical tech-

nique was used.[4, 13, 14, 15, 19, 20] The variation may then have been due to a difference in analytic technique or a difference in selection of normal patients. All the normals used in this study were fasting males, under forty years of age, admitted to the hospital for either the elective repair of an inguinal hernia or a saphenous vein ligation for uncomplicated varicose veins. In some previous studies patients with systemic disease and advanced age were included in the normal group. Since our range of fasting serum iron values was in excellent agreement with those of other workers, it is also possible that our slightly higher average value is due to the relatively small number of normal cases analyzed.

It has been clearly demonstrated that the five-minute specimen of the ten milligram intravenous iron tolerance test represents the TIBC.[5, 14, 16, 17, 18] In contradistinction to the technique used in this study, in vitro measurements of TIBC signify chemical determinations on fasting serum or plasma which reflect the amount of iron binding protein present in the specimen. In our hands, such techniques[18] have been either cumbersome or relatively inaccurate. If, however, excessive quantities of ionizable iron (i.e., 10 mg.) are administered intravenously to a patient, analysis of the serum for iron five minutes after the injection reveals only that quantity of iron bound by the iron binding protein. Repeated injections of 10 mg. of iron fail to raise this five-minute serum iron

TABLE III. PREVIOUS SERUM IRON DETERMINATIONS IN NORMAL MALES

AUTHOR	SEFE (γ%)	TIBC (γ%)	SATURATION (%)
Heilmeyer and Plotner, 1937[6]	126 (81-162)		
Moore, C. V., et al.: J. C. I. **16**: 613-626, 1937	122 (94-174)		
Skouge, E.: Klinische und experimentelle untersuchungen ueber das Serumeisen. Oslo. Dybwad, 173 pp., 1939	118 (79-158)		
Brochner-Mortensen and Olsen, 1940[20]	132 (90-194)		
Vahlquist, 1941[19]	142 (68-263)		
Bjêrre, S., and Christoffersen, N. R.: Nord. Med. **16**: 3314-3317, 1942	111 (72-160)		
Hoyer, K.: Acta Med. Scandinav. **119**: 562-585, 1944	131 (63-189)		
Holmberg, C. G., and Laurell, C. B.: Acta Physiol. Scandinav. **10**: 307-319, 1945*	130 (80-172)	312 (264-366)	43 (23-61)
Cartwright et al., 1946[27]	120 (90-150)		
Laurell, 1947[18]	124 (70-214)	315 (254-406)	36 (21-81)
Greenberg et al., 1947[22]	126 (85-137)	315† (262-392)	40‡
Rath and Finch, 1949[5]	106 (87-147)	311 (254-432)	34 (30-44)
Cartwright and Wintrobe, 1949[16]	127 (79-196)	347 (306-396)	36 (24-49)
Andersson, N. S. E.: Acta Med. Scandinav. **138** (Suppl. 241): 5-71, 1950	115 (74-189)		

*Ten normals (6 M, 4 F).

†In vivo determinations.

‡Based on average values.

SeFe = fasting serum iron; TIBC = total iron binding capacity (in vitro determinations); saturation = SeFe/TIBC ×100.

level above that obtained after the initial iron injection.[16, 26] Our normal TIBC as determined by this in vivo technique was in good agreement with the in vitro measurements presented in Table III. In vitro determinations of the unsaturated iron binding capacity were performed on the five-minute specimens in a number of the normal and abnormal patients. No unsaturated iron-binding protein was found.

It is generally accepted that the serum iron-binding protein is normally about one-third saturated with iron. Our figure of 41 per cent saturation reflects the slightly elevated fasting serum iron level discussed above. Although the serum iron may theoretically vary from zero to about 350 μg per cent (TIBC), it is nevertheless maintained within a comparatively narrow range (75 to 185 μg per cent). It is therefore apparent that certain regulatory mechanisms are operating to maintain approximately one-third saturation of the iron binding protein. The intravenous iron tolerance test loads these mechanisms to their capacity. The decrease in the serum iron level from the peak value of this load reflects the tendency of these mechanisms to effect restitution of the normal serum iron level. This decrease (DEC=TIBC—one-hundred-twenty-minute specimen) was small (about 50 μg per cent), and in normals bore a fairly constant relationship to the size of the load (INC=TIBC—SeFe).

TABLE IV. PREVIOUS TEN MILLIGRAM INTRAVENOUS IRON TOLERANCE TESTS IN NORMAL MALES

AUTHOR	SEFE (γ%)	TIBC (γ%)	120′ (γ%)	SATURATION (%)	INC (γ%)	DEC (γ%)	DEC/INC (OUR CALCULATION)
Heilmeyer and Plotner, 1937[6]*	70	367	40′ spec. 330	19	297	37	0.12
	124	358	40′ spec. 338	35	234	20	0.09
Brochner-Mortensen, 1943[13]*	141 (100-178)	381 (282-453)	259† (246-265)	40 (26-51)	210 (157-333)	73† (52-89)	0.34† (0.31-0.37)
Totterman, 1949[14]*	134 (89-160)	376 (307-406)	337 (282-366)	36 (26-47)	242 (164-267)	40 (25-54)	0.16 (0.10-0.23)
Brendstrup, 1950[17]	81 (50-125)	263 (226-326)	192 (148-240)	30 (20-41)	182 (143-214)	71 (46-118)	0.39 (0.28-0.55)

*Ferrous iron used as loading material.

†Based on three of eight patients.

SeFe = fasting serum iron; TIBC = total iron binding capacity (5′ specimen); saturation = SeFe/TIBC ×100; INC = increase (TIBC—SeFe); DEC = decrease (TIBC—120′).

This constant relationship between the abnormal load (INC) and the restitution mechanism (DEC) is conveniently expressed as the DEC/INC ratio. Therefore, this ratio is the mathematical expression of the manner in which the body responds to the abnormal load. We have found that this ratio is predictable with some accuracy in normal and disease states. It averaged 0.26 for the ten normals in our study. Seven ranged between 0.10 and 0.27. Totterman[14] has performed the only previous study of normal intravenous iron tolerance tests comparable in all respects to ours (Table IV). Of thirteen normal adults, his average DEC/INC ratio was 0.16 (our calculation). Ten fell within 0.14 and 0.19.

Since there is no absorption of serum iron by mature erythrocytes[9, 14, 17, 21] and since the excretion of iron is minimal during the test period,[4, 17] it is obvious that DEC is the result of tissue uptake of the serum iron. In the broad sense such tissue uptake includes any iron leaving the serum and is concerned predominantly with the liver, spleen, and bone marrow. Obviously it is impossible to differentiate that iron entering tissue stores from that utilized in erythropoiesis. Whereas the DEC/INC ratio normally averages 0.26, we have observed that most pathologic conditions are associated with a higher ratio, i.e., an abnormally high tissue uptake in response to the test load. This has been noted in the anemias associated with chronic infection, metastatic malignancy, and chronic blood loss. Chronic infection without anemia may also show this abnormality. This correlates with the work of Greenberg and associates who demonstrated that the rate of disappearance of infused iron from the plasma of patients with infection was about twice that seen in normal subjects.[22] Since the DEC/INC ratio was elevated in many of the pathologic conditions studied, it would seem that in the normal the regulatory mechanisms act to inhibit the rapid uptake of bound iron from the serum. The increased ratio in pathologic states might be the result of some defect in this inhibitory mechanism.

It should be emphasized that the DEC/INC ratio is not an absolute measure of the physiological turnover of serum-tissue iron. This ratio is the expression of the tissue uptake under the stimulus of an abnormally high serum iron level and is not strictly comparable to iron clearance values derived from studies using minute tracer doses of radioactive iron (Fe^{59}).[23, 24] Moreover, the size of the load (INC) is to some extent predetermined by the fasting serum iron level and the TIBC. Theoretically, increased iron storage, associated with an increased saturation of the iron binding protein, might give rise to a high DEC/INC ratio without an absolute change in the tissue uptake of serum iron. This possibility will be discussed in greater detail later.

Heilmeyer and Plotner,[6] in 1937, were the first to perform 10 mg. intravenous iron tolerance tests somewhat similar to those employed in this study. The results of previous investigations utilizing this test in normal subjects are presented in Table IV. Both ferric and ferrous iron were used in these studies. It is unlikely that this difference in loading material significantly affects the results.[14] Nevertheless, these results should not be directly compared with one another or with those of this study because of differences in sampling, analytic technique, and the timing of blood specimens.

To the best of our knowledge only one study of this type has been performed on a patient with hemochromatosis. Frandsen,[4] in 1947, reported two intravenous iron tolerance tests done on one patient with hemochromatosis. In the first test, the SeFe was 211 μg per cent, the TIBC 270 μg per cent and a sixty-minute specimen 219 μg per cent. Our calculation reveals a saturation of 78 per cent. The DEC/INC ratio cannot be calculated for comparison, since no one-hundred-twenty-minute specimen was drawn. In the same patient, seven days later, the SeFe was found to be 262 μg per cent, the TIBC 313 μg per cent, with a resultant calculated saturation of 84 per cent and DEC/INC of 0.84.

The tolerance tests in hemochromatosis as presented in Table II possess the following characteristics: (1) a normal or high SeFe, (2) a normal or low TIBC, (3) a high degree of saturation of the iron binding protein, (4) a normal range for DEC, and (5) a high DEC/INC ratio.

Although the average SeFe in the hemochromatosis group (213 μg per cent) was higher than in the normal group (146 μg per cent) there was considerable overlap. No SeFe value was below the normal range. Four fell within this normal range, and six of the remaining nine were well above it (greater than 256 μg per cent). It is therefore apparent that a normal fasting serum iron value does not exclude the diagnosis of hemochromatosis.

No TIBC value was above the normal range. Five were within this range and eight fell below it. Since cirrhosis of the liver per se is associated with similar TIBC values,[18, 25] it is evident that the TIBC, when considered alone, has no diagnostic significance.

The degree of saturation of the serum iron binding capacity was above the normal range in all the patients with hemochromatosis, although the highest individual normal (58 per cent) did approach the lowest abnormal figure (63 per cent). This elevated serum saturation in hemochromatosis, previously noted by Rath and Finch,[5] is evidently of great diagnostic value in this disease.

Although the absolute figure for DEC in the hemochromatosis group fell within the normal range, the DEC/INC ratio was well above normal in all cases. The patient, P. F., showing the lowest DEC/INC ratios had had over three grams of tissue iron removed by phlebotomy in the six-month period prior to testing. It may be that there was some relationship between this therapy and the low ratios.

Three of the seven patients with hemochromatosis had a finding not as yet noted in any normal subject. This consisted of a fall in the serum iron level by one hundred twenty minutes to a point below the fasting control figure, with a resultant DEC/INC ratio greater than 1.00. In both normal subjects and patients with hemochromatosis, plotting serum iron against time, from the five-minute specimen to that point at which the control serum iron level is reached, results in a hyperboliform curve, with the greatest fall occurring during the first thirty minutes.[17, 22, 26] In normals this control level is not attained before ten hours.[18, 27] In those three cases of hemochromatosis with a DEC/INC ratio greater than 1.00, the initial level was reached in less than sixty minutes.

The abnormally high ratios noted in hemochromatosis would seem to indicate an increased tissue affinity for serum iron under the conditions of the tolerance test. Strictly speaking, these results do not specifically indicate that such excessive uptake occurs at times when the serum iron levels are not artificially increased. Huff et al. studied patients with leukemia, polycythemia, pernicious anemia, and aplastic anemia with minute tracer doses of Fe^{59}.[23] The clearance of radioactive iron from the serum, i.e., tissue uptake or turnover, in these diseases grossly correlated with the DEC/INC ratio in similar conditions studied by means of intravenous iron tolerance tests.[28] This indicates that the DEC/INC ratio may actually reflect tissue uptake not only under the conditions of the test but also at physiologic serum iron levels.

It has been accepted that the specific abnormality in hemochromatosis is an increase in the absorption of iron from the gastrointestinal tract.[29] Our results indicate that in addition to this defect, there may also be an abnormality in the serum iron regulatory mechanisms. In view of the abnormal affinity of the tissues for serum iron and of the mucosal cells for gastrointestinal iron, it is tempting to hypothesize that the two mechanisms have the same underlying pathophysiology.

The abnormalities in the intravenous iron tolerance test noted in patients with hemochromatosis are not specific for this disease. Similar results have been found in pernicious anemia in relapse, Cooley's anemia, acute leukemia, and aplastic anemia.[28] This does not detract from the value of the intravenous iron tolerance test as a diagnostic aid in hemochromatosis, since these other diseases offer no problem in differential diagnosis. The major clinical difficulty is one of differentiation of simple portal cirrhosis with or without diabetes from the cirrhosis associated with hemochromatosis. On the basis of studies now in progress,[25] we feel that the intravenous iron tolerance test is of definite value in differentiating these two conditions. As compared with hemochromatosis, such tests in cirrhosis have revealed: (1) a slightly lower SeFe, (2) a lower degree of saturation, (3) similar TIBC ranges, and (4) a lower DEC/INC ratio. The tendency toward low TIBC values in both diseases might be due to the altered protein metabolism in chronic liver disease, with a specific difficulty in the formation of iron binding protein.

It should be emphasized that a detailed analysis of any single intravenous iron tolerance test must be made to interpret the results properly. Case J. C. illustrates this point. A severely ill patient entered the hospital with the clinical diagnosis of portal cirrhosis, diabetes mellitus, normochromic anemia, and possible hepatoma. The intravenous iron tolerance test (Table II) revealed a normal SeFe, a low TIBC, a high degree of saturation, and a high DEC/INC ratio. An occasional severely ill patient with portal cirrhosis may reveal a somewhat elevated saturation or DEC/INC ratio.[25] A high DEC/INC ratio and low TIBC is also compatible with the presence of malignancy. The diagnosis of hemochromatosis rested upon the fact that there was a high normal SeFe in the face of a moderately severe normochromic anemia. Such an anemia secondary to infection, chronic blood loss, or malignancy is frequently associated with a hypoferremia. Subsequent liver biopsy revealed hemochromatosis and cholangiocarcinoma. This case typifies the necessity for a complete and detailed examination of the results of every iron tolerance test.

There is a theoretical objection against using the DEC/INC ratio to represent the tissue uptake of serum iron in pathologic conditions. Since the load (INC=TIBC—SeFe) is partially determined by the quantity of serum iron binding protein (TIBC), the DEC/INC ratio is obviously related to the state of protein metabolism in the individual patient. Thus a low TIBC with a normal SeFe and DEC would result in a high DEC/INC ratio despite the possibility of normal iron metabolism. The slightly elevated ratio noted in uncomplicated portal cirrhosis[25] may be due to the associated lowered TIBC per se and not to a primary dysfunction of iron metabolism. Hemochromatosis, though

associated with hepatic dysfunction and TIBC values similar to those seen in portal cirrhosis, reveals a much more markedly increased DEC/INC ratio. This partially substantiates the validity of the DEC/INC ratio as a measure of tissue uptake of serum iron but does not obviate the necessity for considering the state of protein metabolism when interpreting a slightly elevated ratio. The correlation of this ratio with the work of Huff and associates,[23] as discussed previously, also supports our feeling that this figure does reflect the state of the body's iron regulatory mechanisms, above and beyond the effect of variations of protein metabolism. At this time, however, it is impossible to state what effect the presence of abnormally large iron stores have upon the tolerance test. It is interesting that at least three conditions associated with hemosiderosis, pernicious anemia, Cooley's anemia, and hemochromatosis, reveal similar abnormalities in the iron tolerance test. Since hemosiderosis is associated with relatively high serum iron values, one might argue that the high DEC/INC ratios seen in these diseases are at least partially due to the low INC (TIBC—SeFe) values. If this is so, one would still find it difficult to explain the increased rate at which the serum iron returned to the fasting values—especially when the one-hundred-twenty-minute specimens occasionally revealed serum iron levels below the initial fasting values.

We have found that the results of the intravenous iron tolerance test may be affected by the presence or absence of acute or chronic liver disease, malignancy, infection, uremia, severely altered protein metabolism, hemosiderosis (either exogenous or endogenous) and various hematologic disorders. Each test must be evaluated in the light of these variable factors, each of which may alter any one or all of the results. It must also be stressed that to interpret the test properly the results must be viewed in their entirety, taking into account the clinical and laboratory data of the individual patient and correlating such data with the various absolute and relative values of the iron tolerance examination.

SUMMARY

The technique of the 10 mg. intravenous iron tolerance test and its results in normal subjects and patients with hemochromatosis are presented.

The salient features of this test in hemochromatosis are: (a) a normal or high fasting serum iron, (b) a normal or low total iron binding capacity, (c) a high degree of saturation of the iron-binding protein, and (d) a high DEC/INC ratio.

The possible significance of the DEC/INC ratio as an expression of the state of the serum iron regulatory mechanisms is presented.

It is suggested that in hemochromatosis there is an increased tissue affinity for serum iron as well as an increased absorption of iron from the gastrointestinal tract.

REFERENCES

1. Beyers, M. R., and Gitlow, S. E.: Metabolism of Iron in Hemochromatosis, Am. J. Clin. Path. **21**: 349-356, 1951.
2. Beyers, M. R., and Gitlow, S. E.: Work in progress.
3. Davis, W. D., and Arrowsmith, W. R.: The Effect of Repeated Bleeding in Hemochromatosis, J. Lab. & Clin. Med. **36**: 814-815, 1950.

4. Frandsen, S.: On the Metabolism of Iron in Hemochromatosis, Acta Med. Scandinav. **128**: 186-201, 1947.
5. Rath, C. E., and Finch, C. A.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation. XXXVIII. Serum Iron Transport. Measurement of Iron Binding Capacity of Serum in Man, J. Clin. Investigation **28**: 79-85, 1949.
6. Heilmeyer, L., and Plotner, K.: Das Serumeisen und die Eisenmangelkrankheit. Jena, 1937, Fischer, 260 pp.
7. Vannotti, A., and Delachaux, A.: Iron Metabolism and Its Clinical Significance, New York, 1949, Grune and Stratton, 254 pp.
8. Knutsen, B.: Hemochromatosis. Case Report With Special Reference to Symptomatology and Pathogenesis, Acta Med. Scandinav. **114**: 280-292, 1943.
9. Moore, C. V., Doan, C. A., and Arrowsmith, W. R.: Studies in Iron Transportation and Metabolism. II. The Mechanism of Iron Transportation: Its Significance in Iron Utilization in Anemic States of Varied Etiology, J. Clin. Investigation **16**: 627-648, 1937.
10. Polack, E.: Ugeshr. f. Laeger. **108**: 638, 1946. As quoted in Frandsen, S.: On the Metabolism of Iron in Hemochromatosis, Acta Med. Scandinav. **128**: 186-201, 1947.
11. Sandelius, G.: Ett fall av hamochromatos, Nord. Med. **17**: 40, 1943.
12. Kristofferson, C. E., and Gripwall, E.: Fall av hamochromatos, Nord. Med. **20**: 2163-2165, 1943.
13. Brochner-Mortensen, K.: Iron Content of the Serum in Patients With Hemorrhagic Anemia, Acta Med. Scandinav. **113**: 345-361, 1943.
14. Totterman, L. E.: Vitamin C and Iron Metabolism, Acta Med. Scandinav. **134** (Suppl. 230): 6-158, 1949.
15. Balle, A.: Om bestemmelse af jern i serum, Nord. Med. **16**: 3543-3544, 1942.
16. Cartwright, G. E., and Wintrobe, M. M.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation. XXXIX. The Anemia of Infection. Studies on the Iron Binding Capacity of Serum, J. Clin. Investigation **28**: 86-99, 1949.
17. Brendstrup, P.: Intravenous Iron Loadings of Patients With Infections, Acta Med. Scandinav. **138** (Suppl. 239): 201-207, 1950.
18. Laurell, C. B.: Studies on the Transportation and Metabolism of Iron in the Body, Acta Physiol. Scandinav. **14** (Suppl. 46): 1-125, 1947.
19. Vahlquist, B. C.: Das serumeisen eine padiatrischklinische und experimentelle studie, Acta Paediat. **28** (Suppl. 5): 1-374 and 1-68, 1941.
20. Brochner-Mortensen, K., and Olsen, C.: Kolimetrisk metode til bestemmelse af jern i serum, Nord. Med. **8**: 2502-2506, 1940.
21. Hahn, P. R., Bale, W. F., Hettig, R. A., and Whipple, G. H.: Radioiron in Plasma Does Not Exchange With Hemoglobin Iron in Red Cells, Science **92**: 131-132, 1940.
22. Greenberg, G. R., Ashenbrucker, H., Lauritsen, M., and Wintrobe, M. M.: The Anemia of Infection. IV. The Lack of Relationship Between the Diversion of Iron From the Plasma and the Origin of the Anemia, J. Clin. Investigation **26**: 114-125, 1947.
23. Huff, R. L., Hennessy, T. G., Austin, R. E., Garcia, J. F., Roberts, B. M., and Lawrence, J. H.: Plasma and Red Cell Iron Turnover in Normal Subjects and in Patients With Various Hematopoietic Disorders, J. Clin. Investigation **29**: 1041-1052, 1950.
24. Rashkoff, I., Wasserman, L. R., Leavitt, D., and Mayer, J.: Radioactive Iron Studies in Anemia, Bull. New York Acad. Med. **27**: 387-388, 1951.
25. Gitlow, S. E., and Beyers, M. R.: Metabolism of Iron. II. Intravenous Iron Tolerance Tests in Laennec's Cirrhosis, in progress.
26. Gitlow, S. E., and Beyers, M. R.: Unpublished data.
27. Cartwright, G. E., Lauritsen, M. A., Jones, P. J., Merrill, I. M., and Wintrobe, M. M.: The Anemia of Infection. I. Hypoferremia, Hypercupremia, and Alterations in Porphyrin Metabolism in Patients, J. Clin. Investigation **25**: 65-80, 1946.
28. Gitlow, S. E., and Beyers, M. R.: Metabolism of Iron. III. Intravenous Iron Tolerance Tests in Various Hematologic Disorders, in progress.
29. Granick, S.: Iron Metabolism and Hemochromatosis, Bull. New York Acad. Med. **25**: 403-428, 1949.

THE BLOOD VOLUME IN PORTAL CIRRHOSIS AS DETERMINED BY P^{32} LABELED RED BLOOD CELLS

GRACE M. HYDE, M.D., NATHANIEL I. BERLIN, M.D., ROBERT J. PARSONS, M.D., JOHN H. LAWRENCE, M.D., AND SHIRLEY PORT, B.A.
BERKELEY, CALIF., AND OAKLAND, CALIF.

THE blood volume in portal cirrhosis of the liver has been studied in the past using the dye, Evans Blue (T-1824).[1-3] In general, these studies have shown a plasma hypervolemia and little change in the total red-cell volume. It seemed desirable to study the blood volume in cases of portal cirrhosis using P^{32} labeled red cells, a method which does not have the inherent inaccuracies of the dye methods.[4, 8]

METHODS

The blood volume was determined with P^{32} labeled red blood cells in twenty-eight patients with portal cirrhosis of the liver by the method of Hevesy and Zerahn[5] as modified in this laboratory.[6] The clinical diagnosis was established by a typical history and physical findings such as enlargement of the liver, or liver and spleen, telangiectases, esophageal varices, distended abdominal veins, and ascites with or without dependent edema. The clinical diagnosis was confirmed by abnormal liver function tests, and in sixteen of the twenty-eight cases by biopsy, laparotomy, or autopsy. Cases complicated by other known diseases such as diabetes, infection, congestive failure, or concomitant carcinoma were excluded from this study.

RESULTS AND DISCUSSION

In general, the blood volume was determined soon after the patient entered the hospital and before therapeutic measures had been undertaken. However, two patients required immediate transfusions to combat shock from acute blood loss, and the blood volume was determined in one eight days and the other seventeen days later, at which time it was felt that the plasma volume was stabilized. Both men and women in a wide age group have been studied.

The results are presented in Tables I, II, III, and IV.

For purposes of discussion, the normal range of the plasma volume is considered to be between 32 and 46 ml. per kilogram body weight, and total red cell volume between 24 and 33 ml. per kilogram body weight, as determined by this method.[6, 9]

The patients have been divided into four groups on the basis of their blood volumes (Table I). In Group I there are seven patients who have a low total blood volume and a low plasma volume. Group II is a group similar to Group I, with the exception that the plasma volumes are in the range of normal but below the average. Group III comprises those patients with a high total blood volume, high plasma volume. Group IV comprises those patients

From the Donner Laboratory of Medical Physics, University of California, Berkeley (G. M. H., N. I. B., J. H. L., and S. P.), and the Pathology Department, Highland-Alameda County Hospital, Oakland, Calif. (G. M. H., and R. J. P.).

These studies were supported in part by the Atomic Energy Commission.
Received for publication, Nov. 14, 1951.

with plasma volumes within the normal range but above the average. The majority of the patients with cirrhosis showed a reduced total red-cell volume, only seven (25 per cent) showing a total red-cell volume within the normal range. The average total red-cell volume was 19.5 ml. per kilogram body weight; the range was 13.3 to 29.4 ml. per kilogram body weight.

In Group II and Group IV the plasma volumes were within the range for normal; however, it should be noted that in both groups there was a distinct tendency for the plasma volumes to be near the extremes of the normal range: In Group II, five of the seven patients had plasma volumes within 2 ml. per kilogram of the lower limit and in Group IV, four of the seven fell within these same limits, i.e., were within 2 ml. of the upper limit of normal range. In Group III the plasma volume was above the upper limit of normal in all cases, and Group I below the lower limit of normal.

In one patient, No. 19 (see Table II), the blood volume determined six and fifteen months after the initial study showed a complete reversal from a high normal blood volume with a normal red cell volume and slightly elevated plasma volume to a low blood volume, and a low total red-cell volume and a low normal plasma volume. During these intervals, the patient had been at home and continued to indulge excessively in alcohol. During the second interval there was both a striking weight and blood volume decrease. However, the extent of the decrease was approximately the same, so that there was no difference noted in the blood volume in terms of milliliters per kilogram body weight. Clinically the course of the disease during this time had been progressively downhill. In contrast to this, two other patients (Nos. 2 and 25, Table III), who were in the hospital for a considerable period of time under controlled conditions, showed an increase in the blood volume, due to an increase in the plasma volume. Another patient who had been in the hospital nine months and who had been given supportive therapy with a view to preparing him for surgery (a portocaval anastomosis) had a normal blood volume: 71.2 ml., total red cell volume 33.1, and plasma volume 38.1 ml. per kilogram body weight.

TABLE II. THE CHANGE IN BLOOD VOLUME IN PATIENT NO. 19 DURING RELAPSE

			TOTAL BLOOD VOLUME		TOTAL RED CELL VOLUME		PLASMA VOLUME	
PATIENT	DATE	WEIGHT	(ML.)	(ML./KG. BODY WT.)	(ML.)	(ML./KG. BODY WT.)	(ML.)	(ML./KG. BODY WT.)
19	8/11/50	61.8	4630	74.8	1570	25.4	3050	49.4
	2/15/51	73.9	3730	50.4	1300	17.6	2420	32.6
	11/ 1/51	50.9	2620	51.4	920	18.0	1590	33.0

The number of cases observed is too small to draw any conclusions, but further studies are contemplated to see whether the total blood volume, plasma volume, and total red-cell volume increase during liver regeneration or compensation in a larger series of patients. In this connection it should be mentioned that Labby and Hoagland[7] have shown with T-1824 that during the acute stage of infectious hepatitis there is an increased plasma volume which then decreases with recovery of normal liver function.

TABLE III. THE CHANGE IN BLOOD VOLUME IN PATIENTS NO. 2 AND NO. 25 DURING THERAPY

PATIENT	DATE	WEIGHT	TOTAL BLOOD VOLUME (ML.)	TOTAL BLOOD VOLUME (ML./KG. BODY WT.)	TOTAL RED CELL VOLUME (ML.)	TOTAL RED CELL VOLUME (ML./KG. BODY WT.)	PLASMA VOLUME (ML.)	PLASMA VOLUME (ML./KG. BODY WT.)
2	5/16/50	61.3	2600	42.3	1010	16.5	1580	25.8
	6/13/50	59.5	2930	49.2	910	14.3	1990	33.5
	7/19/50	54.1	3190	59.0	1050	19.5	2140	39.5
25	11/15/50	60.9	4140	67.9	1720	28.2	2360	38.7
	12/ 7/50	58.2	4495	77.2	1800	30.9	2650	45.6

Illustrative of the clinical value of the blood volume in this disease are the following two cases of bleeding from esophageal varices.

Patient 21 entered the hospital with a history of hematemesis. The blood volume was determined soon after entry. The following evening he had a massive hematemesis, clinically estimated to be about 4,000 ml., and went into shock from which he recovered after administration of 500 ml. of plasma. The blood volume was then determined (Table IV) and the clinical impression of blood loss was confirmed. Calculated blood volume loss was 3,350 ml., total red-cell volume loss was 1,190, and plasma volume loss was 2,170. However, the actual blood loss must have been greater than 3,350 ml.; it must be the amount of blood which would contain 1,190 ml. of red cells. Because the hematocrit was changing, this can be estimated only as being approximately 4,800 ml.

TABLE IV. THE CHANGES IN BLOOD VOLUME DUE TO HEMATEMESIS IN PATIENTS NO. 21 AND NO. 16

PATIENT	DATE	TOTAL BLOOD VOLUME (ML.)	TOTAL RED CELL VOLUME (ML.)	PLASMA VOLUME (ML.)	HEMATOCRIT
21	3/14/51	6,390	1,790	4,540	28
	3/16/51	3,540	600	2,870	17
16	3/30/51	6,420	1,930	4,490	30
	5/29/51	5,920	1,300	4,620	22
	(After 3,000 ml. blood and 1,250 ml. plasma)				
	6/ 4/51	6,888	1,930	4,950	28
	(Between May 29, 1951, and June 4, 1951, the patient was given 1,500 ml. of whole blood.)				

The second patient (No. 16) following discharge from the hospital resumed his alcohol intake and later re-entered the hospital in shock from hematemesis on May 27, 1951. The blood volume was not determined until the patient had received 3,000 ml. of blood and 1,250 ml. of plasma. This shows that within the three-day period of blood administration the total red-cell volume was only 100 ml. greater than the amount transfused (1,200 c.c. of red cells). Thus the patient entered the hospital after considerable blood loss and continued to lose blood while receiving the transfusions. The third blood volume shows that after additional transfusions totaling 1,500 ml., the blood volume has been restored to the level found at the time of discharge on the first admission to the hospital. This also shows that between the second and

TABLE I

PATIENT	AGE	HEIGHT	WEIGHT (KG.)	ALCOHOLISM	BLEEDING	R.B.C.	HG IN GM./100 C.C. BLOOD	HEMATOCRIT	TOTAL BLOOD VOLUME (ML./KG.	TOTAL RED CELL VOLUME BODY	PLASMA VOLUME WEIGHT	(GM.
Group I: Low Total Blood Volume, Low Plasma Volume												
1	57	5′ 6″	45.5	chronic	none	–	11.6	37	48.6	17.9	30.1	
2	49	5′ 4″	61.5	chronic	none	3.45	10.1	39	42.3	16.5	25.8	5.6
3	63	4′ 11″	58.2	chronic	none	–	13.9	37	36.9	13.7	23.2	
4	56	5′ 3″	53.2	chronic	none	2.87	9.6	35	44.5	15.5	28.4	4.7
5	43	5′ 4″	59.1	chronic	melena	2.92	8.6	33	44.6	14.7	29.6	
6	60	5′ 8″	102.0	chronic	none	–	15.3	46	57.4	26.4	29.8	5.5
7	41	5′ 7″	111.8	chronic	hematemesis	–	12.7	41	31.1	12.7	18.0	6.3
Group II: Low Total Blood Volume; Normal Plasma Volume—But Below Average												
8	49	5′ 9″	50.4	chronic	none	3.56	11.0	34	50.1	17.0	33.1	6.67
9	42	6′ 1″	74.1	chronic	hematemesis	–	11.6	37	55.9	20.7	35.2	
10	70	5′ 2″	51.7	chronic	none	3.29	10.5	28	47.6	13.3	34.3	5.06
11	40	5′ 1″	64.5	chronic	hematemesis	1.75	6.9	35	51.1	17.8	32.2	6.02
12	41	5′ 7″	57.9	chronic	hematemesis	3.23	10.8	34	51.1	17.4	33.3	
13	52	5′	40.6	chronic	none	3.01	11.9	36	53.2	19.2	33.5	5.52
14	36	5′ 5″	71.8	chronic	epistaxis	3.30	10.1	33	50.7	16.7	33.4	4.70
Group III: High Total Blood Volume, High Plasma Volume												
15*	42	5′ 2″	51.4	chronic	melena	1.62	3.7	19	71.8	13.6	58.2	
16*	32	5′ 7″	77.6	chronic	melena	2.95	8.9	30	82.6	24.8	57.8	6.22
17*	55	5′ 5″	59.1	chronic	none	1.88	7.7	29	78.9	32.2	55.9	
18†	53	5′ 8″	58.3	chronic	hematemesis	–	8.9	26	71.8	18.7	52.4	
19†	40	5′	61.1	chronic	hematemesis	3.16	10.1	34	74.8	25.4	49.4	5.61
20†	71	5′	41.3	–	none	3.66	10.8	23	70.0	16.1	53.2	
21†	46	6′	60.8	chronic	hematemesis	3.40	9.2	28	104.9	29.4	74.5	5.87
Group IV: Normal Total Blood Volume; Normal Plasma Volume—But Above Average												
22	69	5′ 5½″	83.6	chronic	none	3.23	10.0	28	54.8	15.4	39.4	6.82
23	52	5′ 4″	59.1	chronic	epistaxis, hematemesis vaginal	2.63	9.0	25.5	62.2	15.9	45.4	
24	51	5′ 6″	52.7	chronic	none	3.18	10.0	34	69.9	23.7	45.4	5.88
25	67	5′ 6″	60.9	chronic	none	–	12.7	41.5	67.9	28.2	38.7	
26	52	6′	75.9	chronic	none	–	10.3	34	57.8	19.6	37.6	
27	65	5′ 10″	61.3	chronic	none	3.33	9.8	37	71.5	26.4	44.3	
28	67	5′ 6½″	56.3	chronic	occasional melena	–	11.9	38	72.2	27.4	44.1	6.23

*500 c.c. blood eight days prior to determination.
†500 c.c. blood seventeen days prior to determination.

TABLE I

PLASMA VOLUME	WEIGHT	SERUM PROTEINS (GM./100 ML.)		CEPHALIN FLOCCULATION	THYMOL TURBIDITY	ICTERIC INDEX	ASCITES	PERIPHERAL EDEMA	SPLENOMEGALY	ESOPHAGEAL VARICES	HEPATOMEGALY	BIOPSY
30.1	–			–	–	27	–	–	–	–	+	+
25.8	5.64	2.41						+	–	–	–	+
		3.23		–	3.2	–	4+					
23.2	–			3+	10.0	20	2+	–	–	–	+	–
28.4	4.78	2.46		–	–	20	4+	–	–	–	+	+
		2.32										
29.6	–			–	4.5	150	2+	–	?	–	+	
29.8	5.53	2.97		neg.	2.5	70	4+	2+	–	–	+	
		2.56										
18.0	6.35	2.75		4+	–	35	4+	4+	–	questionable	+	
		3.60										
33.1	6.67	2.69		4+	12.0	60	–	–	–	–	+	+
		3.98										
35.2	–			2+	8.5	90	–	–	–	–	+	+
34.3	5.06			4+	–	190	+	–	–	–	+	+
32.2	6.02			neg.	0.8	10	2+	1+	–	–	+	+
33.3	–			–	1.0	6	–	–	–	–	–	
33.5	5.52			4+	8.9	15	4+	1+	–	–	+	
33.4	4.70			4+	–	160	–					
58.2	–			3+	6.8	30	–	–	–	questionable	+	+
57.8	6.22			2+	3.7	20	–	–	–	–	–	–
55.9	–			4+	5.3	–	4+	2+	–	–	+	+
52.4	–			neg.	1.0	2	–	–	–	x-ray	+	–
49.4	5.61	1.99		4+	7.4	10	3+	slight	–	questionable	+	+
		4.52										
53.2	–			neg.	7.5	110	4+	–	–	–	+	+
74.5	5.87			4+	–	25	–	–	–	–	+	–
39.4	6.82	2.37		–	8.5	4	4+	4+	–	–	+	–
		4.45										
45.4				4+	12.2	120	2+	–	–	questionable	+	–
45.4	5.88	2.41		neg.	–	60	4+	–	–	–	+	+
		3.17										
											+	
38.7				3+	7.4	60	4+	3+	–	–	+	+
37.6				4+	3.0	–	3+	–	–	–	+	+
44.3				3+	4.5	4	1+	slight	+	–	+	+
44.1	6.23	3.24		2+	8.0	6	4+	4+	–	–	+	+
		2.99										

third blood volume determination there was no further bleeding, since the difference in total red-cell volume represents a quantity of red blood cells equivalent to that transfused in that period.

The total blood volume, total red-cell volume, and plasma volumes could not be correlated with hepatomegaly, splenomegaly, ascites, hemorrhagic phenomena, serum proteins, icterus index, intensity of the cephalin flocculation, or thymol turbidity.

Of the four groups of patients, there was a distinct tendency toward a larger total red cell volume on the average (average total red cell volume 22.0 ml. per kilogram) in the fourteen patients with plasma volumes above the average, as compared to the fourteen patients with plasma volumes below average (average total red cell volume 17.1 ml. per kilogram). Although these findings could not be correlated with other clinical and laboratory findings, the findings in the small group studied while undergoing therapy suggest the possibility that the changes in total red cell volume and particularly in the plasma volume may be a more sensitive indicator of the extent of impairment of liver function than the other laboratory or clinical evaluations.

It has been previously observed, and Table I shows that the hematocrit, red cell count, and hemoglobin do not give a true picture of the circulating total red-cell volume or plasma volume. If only the routine hematologic determinations such as hemoglobin, red blood cell count, and hematocrit are made, many cases of severe anemia will be considered as being only slightly anemic, and cases with normal red blood cell volume may appear to be severely anemic.

CONCLUSIONS

1. The blood volume of twenty-eight patients with portal cirrhosis of the liver was studied using P^{32} labeled red blood cells.

2. Seven patients had a low total blood volume and plasma volume.

3. Seven patients had a high or normal total blood volume, high plasma volume, and normal or low total red-cell volumes.

4. Seven patients had high or normal total volume, high normal plasma volume, and normal or low total red-cell volumes.

5. Seven patients had normal total red-cell volumes; the other twenty-two patients were anemic by blood volume standards.

6. The total red-cell volume was significantly higher in the group of patients with plasma volumes above average.

7. It is evident that in cases of cirrhosis of the liver the blood volume may be high, normal, or low, the total red-cell volume may be normal or low, and the total volume of red blood cells and of plasma cannot be predicted from the hematocrit. Further work will be undertaken to see if changes in blood, red-cell, and plasma volumes in response to treatment have any prognostic values.

REFERENCES

1. Bateman, J. C., Shorr, H. M., and Elgvin, T.: Hypervolemic Anemia in Cirrhosis, J. Clin. Investigation **28**: 539, 1949.
2. Perera, G. A.: The Plasma Volume in Laennec's Cirrhosis of the Liver, Ann. Int. Med. **24**: 643, 1946.

3. Hiller, G. L., Huffman, E. R., and Levey, S.: Studies in Cirrhosis of the Liver. I. Relationship Between Plasma Volume, Plasma Protein Concentrations and Total Circulating Proteins, J. Clin. Investigation **28**: 322, 1949.
4. Berlin, N. I., and Grossman, L. M.: A Critique of the Use of the Dye Methods for the Determination of Blood Volume and the Concept of Total Body Hematocrit (to be published).
5. Hevesy, G., and Zerahn, K.: Determination of Red Corpuscle Content, Acta Physiol. Scandinav. **4**: 376, 1942.
6. Berlin, N. I., Lawrence, J. H., and Gartland, J.: The Blood Volume in Chronic Leucemia as Determined by P^{32} Labeled Red Blood Cells, J. LAB. & CLIN. MED. **36**: 435, 1950.
7. Labby, D. H., and Hoagland, C. E.: Water Storage and Movements of Body Fluids and Chlorides During Acute Liver Disease, J. Clin. Investigation **26**: 343, 1947.
8. Gregersen, M. I.: Blood Volume, Ann. Rev. Physiol. **13**: 397, 1951.
9. Berlin, N. I., Hyde, G. M., Parsons, R. J., Lawrence, J. H., and Port, S.: Blood Volume of the Normal Female as Determined with P^{32} Labeled Red Blood Cells, Proc. Soc. Exper. Biol. & Med. **76**: 831, 1951.

STUDIES ON THE SEPARATION OF CELL TYPES IN SEROSANGUINOUS FLUIDS, BLOOD, AND VAGINAL FLUIDS BY FLOTATION ON BOVINE PLASMA ALBUMIN

DON W. FAWCETT, M.D.,* AND BERT L. VALLEE, M.D.**
BOSTON, MASS.

IN STUDIES on malarial blood, Ferrebee and Geiman[4] described the separation of parasitized erythrocytes from normal erythrocytes using a method which took advantage of the difference in their specific gravity. The molarial whole blood was layered onto a solution of plasma albumin of high specific gravity. After centrifugation normal red blood cells, having a specific gravity greater than that of the albumin, were packed at the bottom of the tube while the lighter infected cells remained suspended at the interface between plasma and albumin. A modification of this technique has been employed by Vallee, Hughes, and Gibson[10] to effect complete separation of leukocytes from whole blood. The successful application of the same physical principle to the examination of bloody serous fluids for cancer cells has been reported previously by Fawcett, Vallee, and Soule.[3] The present paper presents the results of further experience in the separation of cell types in serous effusions, blood, and vaginal fluids by differential flotation on bovine plasma albumin.

MATERIALS AND METHODS

The observations on serosanguinous fluids are based upon the study of twenty-four pleural and abdominal fluids submitted to the Cytology Laboratory at the Vincent Memorial Hospital for diagnosis. The clinical diagnosis and the source and appearance of the fluids are presented in Table I. For routine examination, a 40 ml. sample of each fluid was centrifuged, and smears of the sediment were fixed and stained by the method of Papanicolaou.[7] Using the remainder of each specimen, an effort was made to segregate its cell types by differential flotation on albumin solutions of different density. Sterile 35 per cent solution of bovine plasma albumin (Armour Laboratories) having a specific gravity of approximately 1.090 was diluted with varying amounts of normal saline to make solutions having the desired specific gravity. Preliminary studies showed that it was possible to eliminate erythrocytes from the sediment of serous fluids by employing an albumin solution with a specific gravity of about 1.050.[3] The following procedure was employed:

1. Two 40 ml. centrifuge tubes are filled with the serous fluid to be examined and centrifuged five minutes at 2,000 to 2,500 r.p.m.

2. The supernatant serous fluid is discarded. The cellular sediment from both tubes is combined in 8 ml. of saline solution and the cells are resuspended by alternately aspirating and expelling the saline with a pipette. The resulting suspension represents a tenfold concentration of the cell population.

From the Department of Anatomy, Harvard Medical School, the Vincent Memorial Hospital, and the Department of Biology, Massachusetts Institute of Technology.

Supported by a grant-in-aid from Armour and Company, Chicago, Ill., and by the American Cancer Society through an allocation from the Institutional Grant to Harvard University.

The clinical material studied was obtained from the Cytology Laboratory of the Department of Pathology and Bacteriology, Massachusetts General Hospital.

Received for publication, July 9, 1951.

*John and Mary Markle Scholar in Medical Science.

**Senior Research Fellow of the Committee on Growth of the National Research Council, supported by the American Cancer Society.

3. Using a No. 18 gauge needle on a 5 ml. syringe, 35 per cent albumin is aspirated from its rubber-capped vaccine bottle up to the 3.4 ml. mark (Fig. 1, *A*). After the tip of the needle is withdrawn from the albumin, it is immersed in normal saline and the plunger is drawn out to the 5 ml. mark. Saline and albumin are mixed by inverting the syringe several times and the mixture is delivered into a 40 ml. round bottomed centrifuge tube.

4. The saline suspension of cells is layered onto the albumin solution with a dropping pipette and great care is exercised to prevent mixing at the interface (Fig. 1, *B*).

5. The tubes are then centrifuged twenty to twenty-five minutes in an SB 1 International centrifuge at 2,000 to 2,500 r.p.m.

6. After centrifugation, the erythrocytes are found on the bottom of the tube. A thin buffy layer containing cancer cells, mesothelial cells, and macrophages floats at the saline-albumin interface (Fig. 1, *C*). This layer is pipetted off into a 15 ml. conical centrifuge tube, and saline is added to the 15 ml. mark.

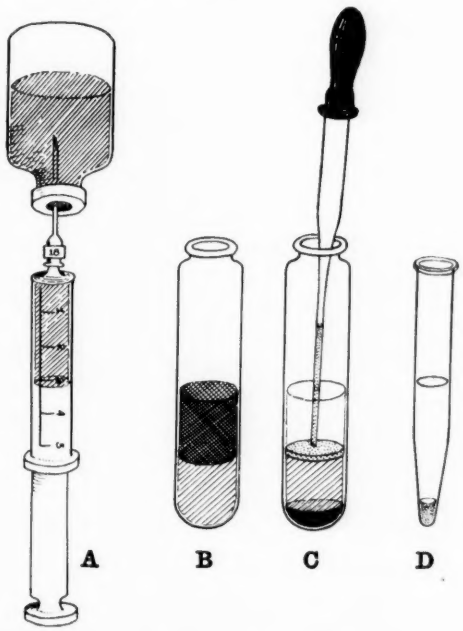


Fig. 1.—Diagram of the principal steps in the procedure for albumin flotation. *A*, Drawing 3.4 c.c. of the viscous 35 per cent albumin into the syringe in which it will be diluted to 5 c.c. with saline. *B*, Suspension of cells layered on top of albumin solution ready for centrifugation. *C*, After centrifugation erythrocytes are packed at the bottom. The lighter cells floating on the surface of the albumin are pipetted off to a small conical centrifuge tube. *D*, The cell pack now free from blood may be studied in smears or by the cell-block method.

7. The cells are centrifuged for five minutes to pack them (Fig. 1, *D*), the supernatant is decanted, and the tube is inverted to drain. Smears of the sediment are made for staining by the Papanicolaou method.

When the accumulation of cells at the end of step 1 in the above procedure was very scant, it was found advisable to decant the supernatant, refill the tubes, and centrifuge again in order to have more cells to work with. When the sediment was not abundant, it was found advantageous to use a 15 ml. conical centrifuge tube in step 3. With 2 to 3 ml. of cells, better results were obtained with the 40 ml. centrifuge bottle in which the saline-albumin interface presented a greater surface area to the falling cells. The addition of saline in step 6 served to wash the cells free of albumin, which would otherwise form an undesirable precipitate when the smears were fixed.

TABLE I

PATIENT	CLINICAL DIAGNOSIS	SOURCE OF FLUID	APPEARANCE OF FLUID	ROUTINE SMEAR	SMEAR AFTER ALBUMIN FLOTATION
018	carcinoma of lung	chest	"grossly bloody"	positive	positive
112	? Hodgkins disease	chest	moderately bloody	negative	doubtful*
116	carcinoma of lung	chest	bloody	doubtful	positive*
118	? carcinoma of lung	chest	cloudy, bloody (8,000 R.B.C.)	negative	negative
146	carcinoma of lung	chest	dark green, bloody	positive	positive
305	portal cirrhosis	abdomen	moderately bloody	negative	negative
324	carcinoma of ovary	abdomen	slightly bloody	positive	positive
356	metastatic adenocarcinoma of stomach	chest	chocolate brown	positive	positive
369	adenocarcinoma of ovary	abdomen	moderately bloody	doubtful	positive*
407	carcinoma of endometrium	abdomen	cloudy bloody	negative	positive*
425	pulmonary infarct congestive failure	chest	blood tinged	negative	negative
534	renal cell carcinoma with pulmonary metastases	chest	"grossly bloody"	negative	positive*
538	carcinoma of lung	chest	moderately bloody	doubtful	positive*
589	metastatic adenocarcinoma	chest	moderately bloody (200,000 R.B.C.)	positive	positive
661	? metastatic hypernephroma	chest	extremely bloody (1,800,000 R.B.C.)	negative	positive*
673	carcinoma of lung	chest	moderately bloody (130,000 R.B.C.)	negative	negative
771	carcinoma of ovary	abdomen	"moderately bloody"	negative	positive*
806	metastatic adenocarcinoma of stomach	chest	moderately bloody	positive	positive
828	bilateral pneumonia	chest	"very bloody"	negative	negative
882	metastatic adenocarcinoma of uterus	chest	moderately bloody	positive	positive
975	metastatic adenocarcinoma of lung	chest	dark brown, bloody	positive	positive
988	carcinoma of lung	chest	"very bloody"	doubtful	positive*
990	adenocarcinoma of uterus	abdomen	blood tinged	positive	positive
995	? carcinoma of lung	chest	very bloody	negative	positive*

*Cases in which the report after albumin flotation differed from the report of the routine smear.

In addition to the study of clinical material, experiments were carried out to determine the amount of blood which could be present and still allow successful separation of cancer cells by albumin flotation. For this purpose artificial cell suspensions were made by adding varying amounts of mouse blood to saline suspensions of cells of the mouse *Ascites tumor*. In these simulated serosanguinous fluids the erythrocytes and tumor cells were counted in a clinical hemocytometer and an effort was then made to separate the cancer cells by means of steps 3 to 7 of the above procedure.

Vaginal fluids from several normal women and from nine patients with carcinoma of the cervix were studied. The fluid collected from the vagina was diluted with 5 ml. of saline. The resulting cell suspension was layered onto an equal volume of albumin in a 15 ml. centrifuge tube. Albumin dilutions used ranged from 2.4 to 3.2 ml. of 35 per cent stock albumin per 5 ml. After fifteen to twenty minutes of centrifugation the cells floating at the saline-albumin interface were washed, collected, and smeared as described above.

Leukocytes were separated from chicken blood by layering 5 ml. of heparinized whole blood onto albumin diluted with saline in the ratio 4.2 ml. to 0.8 ml. After centrifuging for fifteen minutes at 2,000 r.p.m., the leukocytes were pipetted off of the albumin-plasma interface and packed by recentrifuging for five minutes.

RESULTS

Serous Fluids.—Of the twenty-four serosanguinous fluids examined, nineteen were reported positive after elimination of blood by flotation on albumin (Table I). Nine of these had been interpreted as "negative" or as "doubtful" on the basis of routine smears.

If the exfoliated malignant cells were sufficiently numerous, a positive diagnosis could often be made from moderately bloody fluids by the routine method of examining Papanicolaou stained smears of the sediment. A smear prepared in this manner from the bloody pleural fluid of a patient with advanced adenocarcinoma of the lung (589) is shown in Fig. 2*a*. Several single cells and cell groups are present which were adequate for a diagnosis of malignancy. A smear of the cells obtained from this fluid by centrifugation over albumin is presented in Fig. 2*b*. This smear contains several times as many

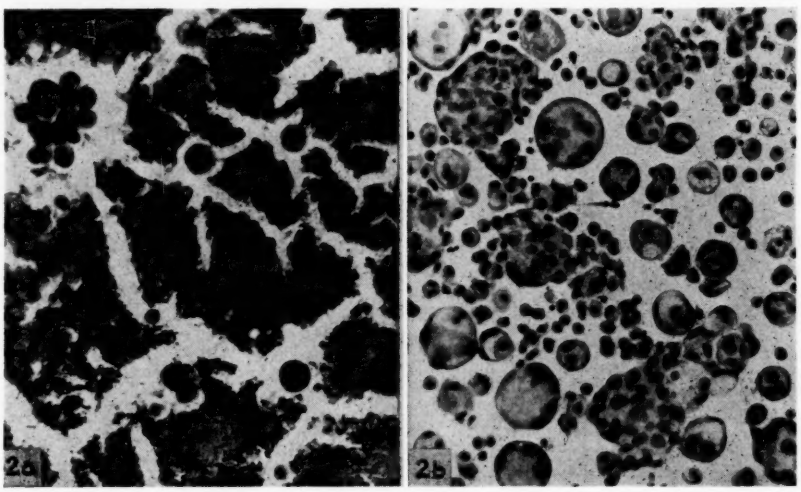


Fig. 2*a*.—Direct smear of the sediment of bloody pleural fluid from a case of metastatic adenocarcinoma. Cancer cells can be identified between the dark masses of blood. (Papanicolaou stain ×200.)

Fig. 2*b*.—Smear prepared from the same fluid after elimination of the blood by albumin flotation. Cancer cells and mesothelial cells are greatly concentrated. (Papanicolaou stain ×200.)

mesothelial cells and large malignant cells as were present in Fig. 2*a*. In this case albumin flotation was not necessary to establish the diagnosis, but a diagnosis can be made from such a smear with greater assurance because it is based upon the examination of a larger number of cells.

When serosanguinous fluids have few cancer cells relative to the number of erythrocytes, the chances of finding and identifying the malignant cells are much diminished. A smear of the sediment of such a fluid from a patient (988) having an adenocarcinoma with pulmonary metastases is shown in Fig. 3*a*. Very few malignant cell groups were present and these were overlain and partially obscured by heavily stained blood and cellular debris. These clumps of cells were regarded as suspicious but could not be seen well enough, nor in sufficient number, to allow a definite diagnosis. The routine smear was there-

fore reported "doubtful." The same fluid when centrifuged over albumin yielded the smear illustrated in Fig. 3*b*. The groups of malignant cells were very conspicuous and a positive diagnosis could be made without hesitation.

In a third example, which illustrates well the advantages of the method, an exceedingly bloody pleural fluid was obtained from a patient who had previously had a nephrectomy for hypernephroma. The hematocrit of this fluid was 17 per cent. Examination of the routine smear revealed only blood and was reported "negative" (Fig. 4*a*). By albumin flotation it was possible to separate a buffy layer of cells consisting of a few macrophages and mesothelial cells and many large groups of malignant cells (Fig. 4*b*). When examined in wet smears, the cells in these groups were heavily laden with refractile lipid droplets, as might be expected of cells exfoliated from a metastatic hypernephroma. In this instance a fluid which was reported negative both in smear and cell block was unequivocally positive after albumin flotation.

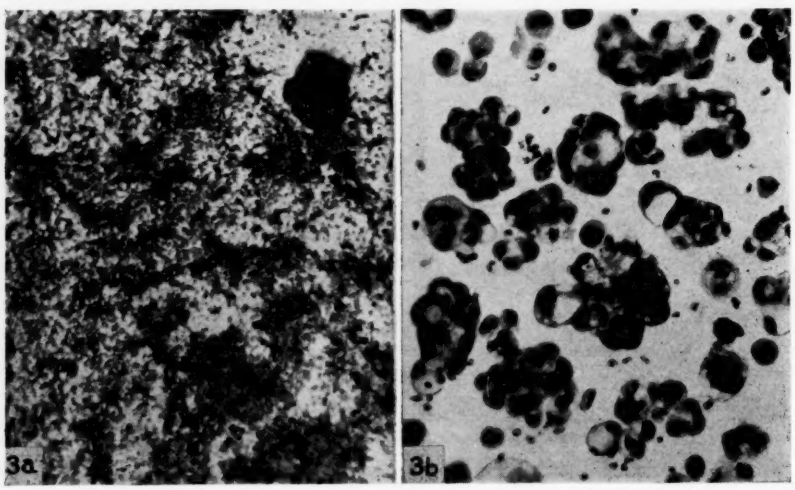


Fig. 3*a*.—Routine smear of a very bloody pleural fluid reported "doubtful." Groups of cells such as that in the upper right were regarded with suspicion, but these were usually obscured by an overlay of heavily stained blood. (Papanicolaou stain ×200.)

Fig. 3*b*.—Smear from the same fluid after albumin flotation. Many large groups of adenocarcinoma cells are clearly visible. (Papanicolaou stain ×200.)

In experiments undertaken to determine approximately how much blood could be present and still permit segregation of the cancer cells by albumin flotation, it was found that mouse *Ascites tumor* cells could regularly be separated, with very little contamination by blood, from suspensions containing about 350 tumor cells and 1,650,000 erythrocytes per cubic millimeter, a ratio of approximately 1 to 5,000.

Theoretically it should be possible to separate any two cell types differing in specific gravity. The difference in specific gravity need not be great, provided the cell types being separated are quite uniform in size and shape. Thus, equal numbers of chicken and rabbit erythrocytes in saline suspension can be separated by finding a concentration of albumin which will arrest the slightly

lighter rabbit cells at the interface while permitting the nucleated chicken cells to pass through. Attempts to separate cancer cells from mesothelial cells and macrophages in serous fluids generally met with little success because these cells differed relatively little in specific gravity, and the cells of any one type varied considerably in size and in state of preservation. In certain instances when adenocarcinoma cells were unusually large or when they contained large watery or lipoidal vacuoles, they were very much lighter than most mesothelial cells and macrophages. Under these circumstances an albumin layer of appropriate density could be found by trial and error which would segregate cancer cells in very nearly pure form. For example, in the case of the hypernephroma cited above (661) it was expected that the specific

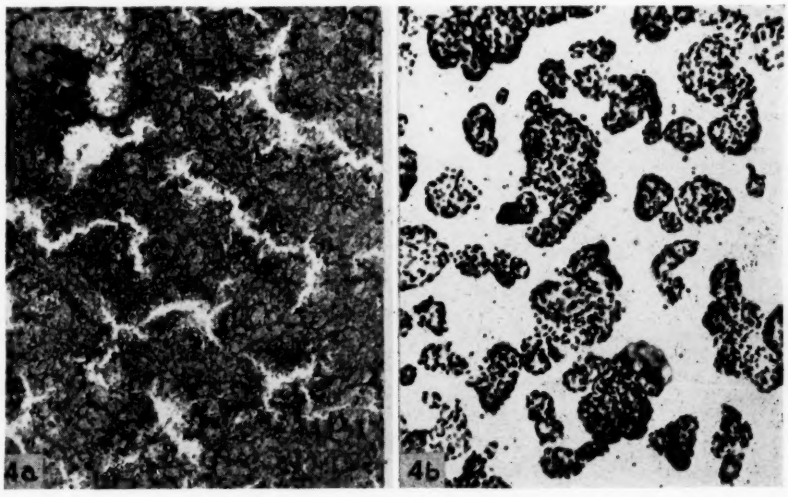


Fig. 4*a*.—A direct smear of sediment from a pleural fluid which contained approximately 1.8 million erythrocytes per cubic millimeter. Routine smears and cell block were both reported "negative." (Papanicolaou stain ×170.)

Fig. 4*b*.—A smear made from this fluid after elimination of blood and mesothelial cells consists almost entirely of groups of hypernephroma cells. (Papanicolaou stain ×170.)

gravity of the malignant cell groups would be unusually low owing to their lipid content. After elimination of erythrocytes from the original sediment by flotation, the cellular material obtained was resuspended in saline and centrifuged, this time using an albumin barrier which was much less dense than the one used routinely (albumin was diluted with saline in the proportions 2.2/2.8 instead of the usual 3.2/1.8). The specific density of this solution was such as to allow the heavier mesothelial cells and macrophages to pass through to the bottom of the tube while the groups of lipid-laden tumor cells formed a layer on the surface of the albumin. In this way a smear consisting entirely of groups of hypernephroma cells was obtained (Fig. 4*b*). The sediment of a few of the serous fluids studied was very rich in macrophages filled with lipid vacuoles. In these instances it was possible to take advantage of their unusually low specific gravity to separate them from the other cellular elements.

Vaginal Fluids.—It was thought that the principle of albumin flotation might be applicable to the segregation of the cell types desquamated in the female reproductive tract. Of the various cellular elements commonly encountered in vaginal fluids, the squamous cells, bacteria, and erythrocytes were heavy enough to pass through the albumin and pack at the bottom of the centrifuge tube, while leukocytes, macrophages, and trichomonads generally floated at the saline-albumin interface (Figs. 5 and 6). The cancer cells in vaginal fluids varied over a wide range of specific gravities. The more undifferentiated tumor cells tended to remain with the lighter leukocyte and macrophage layer. On the other hand the more highly differentiated cells, resembling normal squamous cells, often passed through the albumin.

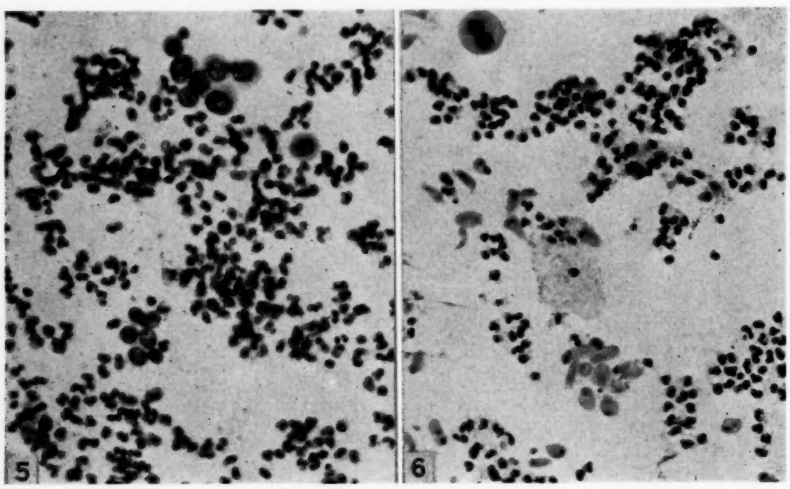


Fig. 5.—Smear of cells from a vaginal fluid which was floated on albumin solution (albumin 2.4 c.c./saline 2.6 c.c.). Leukocytes and two groups of undifferentiated carcinoma cells are shown. Squamous cells have been largely eliminated. (Papanicolaou stain ×275.)

Fig. 6.—Smear of material from vaginal fluid centrifuged over albumin solution (albumin 3.2 c.c./saline 1.8 c.c.). Trichomonads, leukocytes and occasional squamous cells are present. (Papanicolaou stain ×275.)

The cell types in vaginal fluids could not be separated as satisfactorily as those found in blood or bloody serous fluids. Leukocytes often adhered to sheets of squamous cells, and single squamous cells were enclosed by clumps of leukocytes. Many of these aggregations persisted when the cells were suspended in saline. Therefore, the different cell types did not always migrate in the centrifugal field according to their own specific density. A lighter cell could be carried down by a heavier one to which it adhered, and conversely a heavy cell could be held up by several adherent lighter ones. Thus a certain number of leukocytes were commonly carried through the albumin by squamous cells and a few squamous cells were often impounded among the lighter cells at the saline-albumin interface. The presence of even moderate amounts of cervical mucus seriously interfered with separation of cell types because it bound the cells together and prevented them from distributing themselves according to their specific gravities.

Segregation of Leukocytes From Blood.—Albumin flotation was successfully used by Vallee, Hughes, and Gibson,[10] for separating leukocytes from human blood for chemical analysis. In that work the viability of the leukocytes was not an important consideration. In the present investigation leukocytes segregated from chicken blood by albumin flotation were found to be viable and quite suitable for use in tissue culture. This method has proved very valuable in studies on chicken macrophages in vitro which will be reported in detail elsewhere.[11] The starting material for macrophage cultures heretofore has been obtained by removing the clear plasma from centrifuged blood and clotting the buffy coat by addition of thrombin. Explants were then cut from the coagulated buffy coat.

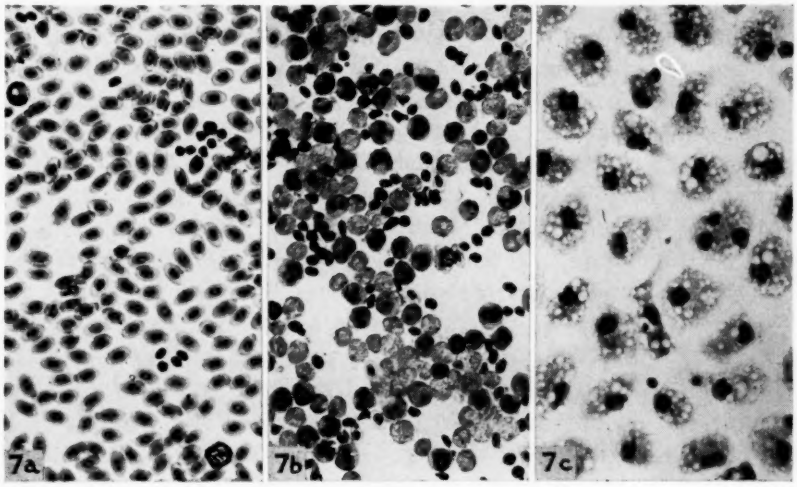


Fig. 7*a*.—A smear of normal chicken peripheral blood showing two leukocytes and several clusters of thrombocytes. (Wright's stain ×400.)

Fig. 7*b*.—A smear of the leukocytes separated from this blood by albumin flotation. (Wright's stain ×400.)

Fig. 7*c*.—Macrophages which had developed after cultivation of those leukocytes in vitro for three days. (May-Grunwald-Giemsa stain ×400.)

In this procedure many erythrocytes may be incorporated in the clot and their subsequent degeneration has a deleterious effect upon the cultures. The macrophages become so heavily laden with phagocytized erythrocytes that they are not suitable for cytological or cytochemical study. This disadvantage was overcome by using leukocytes separated by flotation on albumin. In this procedure leukocytes appeared to incur little or no damage and they were obtained with minimal erythrocyte contamination. When cultured, the monocytes were rapidly transformed into macrophages which were maintained in vitro for two weeks. The degree of concentration achieved may be appreciated by comparing the number of leukocytes in a stained smear of chicken blood (Fig. 7*a*) with the number in a smear of leukocytes concentrated from the same blood by flotation on albumin (Fig. 7*b*). A photomicrograph of the macrophages derived from this material after forty-two hours of cultivation is presented in Fig. 7*c*.

It was also possible to recentrifuge a plasma suspension of these leukocytes over an albumin-saline mixture of lower specific density (3.2/1.8), and thereby

largely eliminate the heavier polymorphonuclear eosinophils, further concentrating the mononuclear elements, which are the only ones capable of prolonged survival in vitro.

DISCUSSION

In diagnostic laboratories interested in the detection of cancer cells in serous fluids, the cell-pack obtained by centrifugation is either embedded in paraffin and sectioned, or it is smeared directly onto slides which are then fixed and stained by the method of Papanicolaou. Whichever method is used, the discovery of a few malignant cells among a great many normal cell types requires prolonged microscopic study by a skilled technician. Extremely bloody fluids present a particularly difficult diagnostic problem. Large numbers of erythrocytes not only cover and obscure the cancer cells but in effect dilute them so that the probability of tumor cells being included in the smear or section examined is diminished. Therefore any method which will eliminate red blood cells from these fluids will result in a relative concentration of the malignant cells and improve the accuracy of diagnosis. The lysis of blood cells by treatment with 3 per cent acetic acid has been recommended as a useful procedure in the study of moderately bloody serous fluids by Saltenspiel,[8] and by Olsen and Bourgeois[6] for bloody vaginal fluids. However, the acid, in addition to its destructive action upon erythrocytes, may alter the morphology and staining reactions of the cancer cells, and thus interfere with their identification.

The use of unphysiological solutions is avoided by centrifugation over an iso-osmotic solution of albumin as described here, and cancer cells, mesothelial cells, leukocytes, and macrophages can be recovered from extremely bloody fluids in which these cell types together constitute only a small fraction of the total cell pack. In several instances this elimination of blood has resulted in a positive diagnosis where other methods have failed. Hence albumin flotation is regarded as a valuable ancillary procedure for the handling of those very bloody specimens which are most difficult to diagnose by the methods now in common use.

Albumin flotation is not recommended in the study of clear serous fluids uncontaminated by blood. Separation of a single cell type from an admixture of macrophages, mesothelial cells, and cancer cells is theoretically possible, but because the differences in specific gravity of these cells are small and the range of variation among cells of any one type is large, such refined separations are too uncertain and complicated to be practical for routine diagnostic purposes. In a few favorable cases nearly pure populations of macrophages and of cancer cells have been obtained and the method holds considerable promise as a research tool in special projects requiring relatively pure suspensions of a single cell type.

The distribution of cells after centrifugation over solutions of albumin has been thought to depend principally upon the relative specific gravity of the cells, the density and osmotic pressure of the solutions involved.[9] There is reason to believe, however, that other factors are involved, since it was found that cells which would float on albumin of a certain specific density were not

held up in the centrifugal field by a sucrose solution of the same calculated specific density. While this may be an effect of the osmotic pressure of the sucrose, it is noteworthy that the sucrose solution is less viscous than the corresponding albumin solution. Hence, it is possible that the results obtained with any given solution of albumin depend to some extent upon its high viscosity as well as its specific gravity.

Efforts to adapt the method for use with vaginal fluids were somewhat disappointing. In those few instances where direct smears of a copious, grossly bloody or purulent discharge were unsatisfactory, centrifugation over albumin facilitated the diagnosis by eliminating blood cells and most of the squamous epithelial cells from the smear. However, in the majority of vaginal fluid specimens, albumin flotation was of doubtful value owing (1) to the small amount of fluid available, (2) the variability in specific density of squamous carcinoma cells, (3) the tendency for cells of different type to cohere, and (4) the difficulties resulting from the presence of cervical mucus. It is possible that some of these disadvantages could be overcome by further experimentation with mucolytic agents and methods for bringing about the dissociation of cells.

The viability of leukocytes separated from whole blood by albumin flotation has been questioned by Buckley, Powell, and Gibson,[1] who prefer the method of Minor and Burnett,[5] which employs fibrinogen to accelerate erythrocyte sedimentation, leaving the leukocytes suspended in plasma enriched with added fibrinogen. It was contended that clumping of leukocytes at the plasma-albumin interface and rise of temperature during centrifugation might be inimical to their survival. Actually, the viability of chicken leukocytes separated by albumin flotation has been clearly demonstrated by their capacity to survive in vitro and give rise to pure cultures of macrophages.[11] The duration of centrifugation which has been employed for chicken blood (fifteen minutes) is no greater than that required for obtaining the buffy coat of blood as originally recommended for tissue cultures of leukocytes by Carrel and Ebeling.[2] In this short time it is unlikely that the cells are ever brought above body temperature. The tendency for the leukocytes to adhere when concentrated by centrifugation over albumin makes it difficult to prepare smears with a uniform distribution of cells for cytological study; it does not, however, interfere with the preparation of cultures. When incubated, the leukocytes readily disengage themselves from the cell clumps and wander out into the culture medium.

SUMMARY

A procedure for the elimination of blood from the sediment of serosanguinous fluids is described. The method involves differential centrifugation with an iso-osmotic solution of bovine albumin adjusted in specific gravity to allow cancer cells, mesothelial cells and macrophages to float while the heavier erythrocytes are packed at the bottom of the tube.

Of twenty-four serosanguinous fluids studied by flotation on albumin, nineteen were found to contain cancer cells. Nine of these "positive" fluids had

been reported "negative" or "doubtful" in routine smears and one which was reported as "negative" by routine smear was considered "doubtful" after flotation. Hence albumin flotation is regarded as a useful procedure in the handling of excessively bloody serous fluids which are difficult to examine in direct smears or cell-block preparations. It is occasionally useful in the examination of copious, bloody vaginal fluids.

The method is also applicable to the separation of leukocytes from whole blood for tissue culture, and to various fundamental problems in which relatively pure populations of a single cell type are required.

REFERENCES

1. Buckley, E. S., Powell, M. J., and Gibson, H. J. G.: The Separation of the Formed Elements of Whole Blood by Means of Fraction I, J. LAB. & CLIN. MED. **36**: 29-39, 1950.
2. Carrel, A., and Ebeling, A. H.: Pure Cultures of Large Mononuclear Leucocytes, J. Exper. Med. **36**: 365-379, 1922.
3. Fawcett, D. W., Vallee, B. L., and Soule, M. H.: A Method for Concentration and Segregation of Malignant Cells From Bloody Pleural and Peritoneal Fluids, Science **111**: 34-36, 1950.
4. Ferrebee, J. W., and Geiman, Q. M.: Studies on Malarial Parasites. III. A Procedure for Preparing Concentrates of Plasmodium Vivax, J. Infect. Dis. **78**: 173-179, 1946.
5. Minor, A. H., and Burnett, L.: A Method for Obtaining Living Leucocytes From Human Peripheral Blood by Acceleration of Erythrocytes Sedimentation, Blood **3**: 799, 1948.
6. Olsen, M., and Bourgeois, G. A.: A Technique for Hemolyzing Erythrocytes and Erythrocytic Debris in the Bloody Vaginal Smear, J. LAB. & CLIN. MED. **36**: 766-768, 1950.
7. Papanicolaou, G. N.: A New Procedure for Staining Vaginal Smears, Science **95**: 538-539, 1942.
8. Saltenspiel, E.: Cytological Diagnosis of Cancer in Transudates and Exudates, Surg., Gynec. & Obst. **89**: 478-484, 1949.
9. Spear, F.: Separation of Leucocytes From Whole Blood by Flotation on Gum Acacia, Blood **3**: 1055-1056, 1948.
10. Vallee, B. L., Hughes, W. L., and Gibson, J. B.: A Method for the Separation of Leucocytes From Whole Blood by Flotation on Serum Albumin; Blood, Special Issue, **1**: 82-87, 1947.
11. Weiss, L., and Fawcett, D. W.: Cytochemical Observations on Chicken Monocytes, Macrophages, and Giant Cells in Tissue Culture, To be published.

THE RESPONSE OF MONOCYTES TO ADRENAL CORTICAL EXTRACT*

EDNA H. TOMPKINS, M.D.
BOSTON, MASS.

INTRAVENOUS injection of rabbits with sphingomyelins[24] has been shown to elicit responses of the circulating lymphocytes and heterophils that are identical to those reported following injections of mice, rats and dogs with ACTH or with adrenal cortical hormones.[5, 19, 25] The lymphocytes decrease within the same interval of time after injection of sphingomyelins as after injection of the hormones, remain diminished for the same duration, and overcompensate in the same manner. The heterophils increase slightly, and the total white counts diminish or remain unchanged. While probably related in some manner to that obtained with ACTH, this reaction is considered specific to sphingomyelins, since control injections of the experimental menstruum, or of a different phospholipid, failed to elicit comparable responses of the blood cells. Interestingly, the response of the monocytes to sphingomyelins parallels that of the lymphocytes in all respects. The differential counts were performed by the supravital technique, which is particularly helpful in the differentiation of monocytes; and rabbits tend normally to have appreciable numbers of these cells. This combination of factors aided in emphasizing the parallelism between the reactions of the blood monocytes and lymphocytes to these lipids.

Specific separation of monocytes from lymphocytes was not indicated in the studies concerned with the action of adrenal cortical hormones on lymphocytes.[5, 6, 8, 13, 16] It seemed, therefore, in view of this fact, and of the parallelism between the reaction of lymphocytes to sphingomyelins and to adrenal cortical hormones, and between the reaction of both lymphocytes and monocytes to sphingomyelins, that investigation of the action of adrenal cortical hormones on monocytes was also indicated. This seemed particularly pertinent in view of the concept of lymphocytes as the locus for the formation of antibodies which developed from the action of adrenal cortical hormones, and from various immunologic studies, upon those cells.[7, 8, 13, 14, 15, 16, 25] This concept is opposed by the contemporary one of "young plasma cells" as the locus of this activity.[1, 2, 9, 10, 17] Both concepts are alike, however, in that they are based on presumptive reasoning, and in that the proponents of each have failed to specify separation of monocytes, young cells of the reticulo-endothelial system, which has long been regarded as the site of antibody formation,[18] from the cells which they implicate. Investigation of the reaction of monocytes under any of the experimental conditions employed by these workers, therefore, seemed to promise,

From the Cancer Research Institute, New England Deaconess Hospital, Boston, Mass.
Received for publication, Aug. 23, 1951.

*The experimental data for this paper were obtained while the author was a member of the Laboratory of Applied Physiology, Yale University, New Haven, Conn. Compilation was performed under U. S. Atomic Energy Commission Contract AT(30-1)-901 with the New England Deaconess Hospital, Boston, Mass.

in addition to the specific information sought, beginning light in the confusion that has arisen in recent years concerning the cytologic locus for the formation of antibodies.

EXPERIMENTAL PROCEDURE

Only four rabbits were used for this investigation, two males (Nos. 50 and 51) and two females (Nos. 44 and 49), which had long been habituated to the procedures of the laboratory. One rabbit (No. 49) with an exceptionally high basic count of monocytes was chosen purposely in order to permit of exaggeration of any effects that might occur in those cells. Injections were given both subcutaneously and intravenously in this animal. Otherwise all injections were given intravenously. Wilson's aqueous extract of adrenal cortex* was used throughout, without dilution, 5 ml. per rabbit intravenously, and 7 ml. subcutaneously. The rabbits' weights were as follows: No. 44, 5.9 kilograms; No. 49, 3.7 kilograms; No. 50, 5.2 kilograms; and No. 51, 3.1 kilograms.

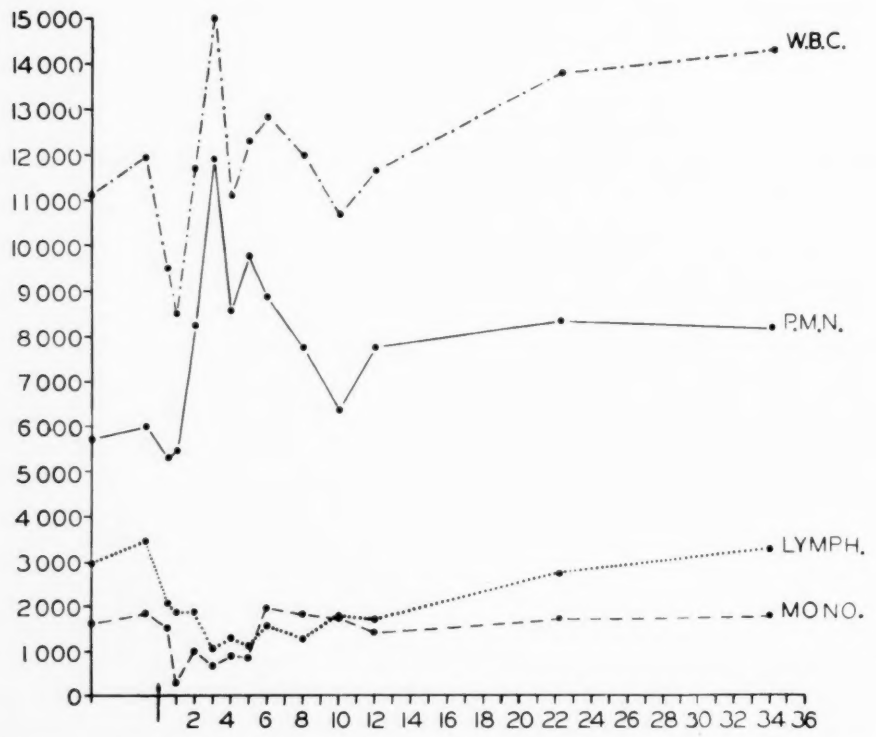


Fig. 1.—Rabbit 49. Counts of the white blood cells following intravenous injection of 5 c.c. of adrenal cortical extract. Abscissa—hours; ordinate—absolute numbers of cells.

The blood was obtained by a prick of an ear vein. Trenner automatic white blood pipettes were filled in triplicate. The counts were made in duplicate and the third pipette used only when they disagreed. The differential counts of 200 cells were made supravitally in order to facilitate differentiation of monocytes. All values are expressed in absolute numbers.

Control injections of other than the experimental extract were not made, because the control studies which were carried out during the course of various investigations concerned

*This extract was kindly secured for us through the courtesy of Dr. David Klein, Wilson Laboratories, Chicago, Ill.

with the reaction of rabbits to lipids[23, 24] seem to serve adequately for the present studies also. They demonstrate that the reactions of the blood cells following control injections of rabbits habituated to the laboratory procedures and handled considerately throughout the experimental period are in no way equivalent to those following adrenal cortical stimulation. The routine in this experiment was similar in every respect to that used in the former studies. Each rabbit did serve as its own control, however, since two or more counts were made at hourly intervals before injection of the extract. The injection was given only when a steady control level was assured.

Counts were made at hourly intervals following injections for the first six hours, at two-hour intervals thereafter for such periods as seemed indicated, and then every twelve hours for several days. The counts of basophils and eosinophils entered into the computations, but have been omitted from the records for purposes of simplicity since they show no consistent changes. The absolute counts in the published data, therefore, obviously never add to the values of the total white blood counts.

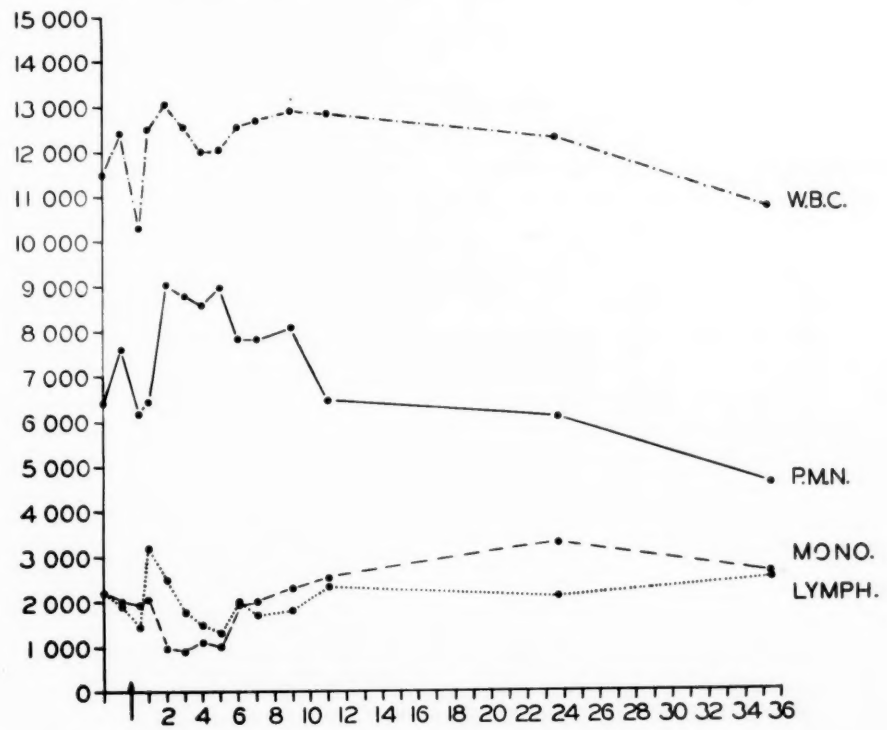


Fig. 2.—Same rabbit and same coordinates as Fig. 1. Counts following subcutaneous injection of 7 c.c. of adrenal cortical extract. Note the similarity of the reaction of the monocytes to their reaction following intravenous injection of the extract (Fig. 1).

RESULTS

The data are presented in Tables I and II. Table I records the absolute numbers of the different white blood cells of the control values, and of each count following the injections. The control values represent the average of the counts made before injection. Table II was compiled from Table I. It records the intervals of time after injection at which maximal increases or decreases occurred in the various cells, and presents the magnitude of these changes as

TABLE I. THE REACTION OF THE WHITE BLOOD CELLS TO INTRAVENOUS INJECTIONS OF ADRENAL CORTICAL EXTRACTS (ABSOLUTE NUMBERS IN FRACTIONS OF 1,000)

NEAREST HOUR AFTER THE INJECTION	NUMBER OF RABBIT															
	44	49	50	51	44	49	50	51	44	49	50	51	44	49	50	51
	TOTAL WHITE BLOOD COUNT				HETEROPHILS				LYMPHOCYTES				MONOCYTES			
Before injection	5.5	11.5 11.9*	8.9	8.4	3.1	5.9 7.0*	4.1	4.8	1.5	3.2 2.1*	3.8	2.4	0.5	1.7 2.1*	0.7	0.6
½	5.9	9.5 10.3	10.0	9.2	3.4	5.3 6.2	6.2	6.0	1.3	2.0 1.4	2.5	1.8	0.7	1.5 1.9	0.8	0.6
1	5.9	8.5 12.5	8.7	8.1	4.1	5.5 6.4	5.9	5.1	1.2	1.8 3.2	1.8	2.4	0.2	0.3 2.1	0.6	0.2
2	5.6	11.7 13.0	6.8	8.4	4.3	8.2 9.1	5.4	6.6	0.9	1.9 2.5	1.1	1.3	0.2	1.0 1.0	0.2	0.3
3	5.5	15.0 12.6	8.1	7.9	3.9	11.9 8.8	6.1	6.0	1.1	1.0 1.8	1.6	1.5	0.2	0.7 0.9	0.2	0.1
4	6.7	11.1 12.0	8.6	6.8	5.2	8.6 8.6	6.4	5.0	0.8	1.3 1.5	1.7	1.3	0.4	0.9 1.1	0.3	0.1
5	6.1	12.3 12.0	7.5	7.4	4.5	9.8 9.0	4.7	5.0	0.8	1.1 1.3	1.8	1.5	0.6	0.9 1.0	0.4	0.4
6	7.0	12.8 12.6	7.5	6.4	4.6	8.9 7.9	4.8	4.2	1.5	1.5 2.0	2.2	1.7	0.6	1.9 1.9	0.3	0.2
8	6.0	12.0 12.7		6.6	4.1	7.7 7.8		4.2	0.7	1.3 1.7		2.0	0.8	1.8 2.0		0.3
10		10.7 12.9	10.4	7.1		6.4 8.1	4.9	3.8		1.8 1.8	4.5	2.3		1.7 2.3	0.7	0.5
12	7.0	11.7 12.9	8.0	7.9	3.9	7.8 6.5	4.1	3.8	2.0	1.7 2.3	3.1	2.8	0.8	1.4 2.5	0.6	0.6
14	5.4				3.0				1.4				1.0			
22		13.9 12.3		9.2		8.4 6.1		5.0		2.7 2.1		3.0		1.7 3.3		0.6
24	4.8		12.2		2.0		5.0		1.9		6.0		0.5		0.9	
36	5.8	14.4 10.9	8.1	8.7	2.9	8.3 4.6	3.5	4.5	1.8	3.3 2.5	3.5	3.1	0.8	1.8 2.6	0.5	0.6
48	5.7	12.9 12.1	11.2	9.2	2.4	7.3 6.0	4.6	4.6	2.0	2.2 2.6	5.6	3.3	0.9	2.4 2.7	0.7	0.7
60	5.5		8.3	9.8	2.8		3.8	4.9	1.6		3.6	3.1	0.8		0.7	0.8
72		13.0	8.1	9.5		6.3	3.0	5.3		2.7	3.3	2.5		2.9	1.6	0.7
84			7.1	8.3			3.1	4.1			3.0	2.5			0.9	0.7
96			11.2	8.9			4.3	4.5			5.4	2.9			1.1	0.7
120	6.1	9.6 11.0			2.7	4.7 5.5			2.2	2.5 2.5			0.7	1.7 1.7		

*The injection in this experiment was given subcutaneously.

percentages of the control values. All counts up to the first thirty-six hours after injection of rabbit 49 are also recorded in Figs. 1 and 2 in order to depict graphically the characteristic curve of reaction, and to illustrate the similarity of reactions with different routes of administration. Fig. 2 is the more typical.

TABLE II. PERCENTAGE DEVIATIONS FROM THE CONTROL VALUES OF THE MAXIMAL CHANGE FOLLOWING INJECTION AND TIME OF ITS OCCURRENCE IN RELATION TO THE TIME OF INJECTION

RABBIT NO.	TOTAL WHITE BLOOD COUNT				HETEROPHILS				LYMPHOCYTES				MONOCYTES			
	0-9 HR.		9 HR. PLUS		0-9 HR.		9 HR. PLUS		0-9 HR.		9 HR. PLUS		0-9 HR.		9 HR. PLUS	
	%	HR.	%	HR.	%	HR.	%	HR.	%	HR.	%	HR.	%	HR.	%	HR.
44	+27	6	−13	24	+68	4	−36	24	−53	8	+47	120	−60	1	+100	14
49	+30	3	+25	36	+102	3	+41	36	−69	3	+3	36	−82	1	+71	72
*	+9	2	−8	36	+30	2	−34	36	−38	5	+24	48	−57	3	+57	22
50	−24	2	+37	24	+56	4	−27	72	−71	2	+58	24	−71	2	+129	72
51	−24	6	+17	60	+38	2	−21	10	−46	2	+38	48	−83	3	+33	60

*The injection in this experiment was given subcutaneously.

The data summarized in Table II are consistent and permit definite conclusions. Both the lymphocytes and monocytes decrease 50 per cent or more within a few hours of the injection of adrenal extract. The heterophils, on the other hand, increase to about the same degree during this time. The lymphocytes begin to return into circulation at about eleven hours after the injection, and gradually reach a peak of about 40 per cent above the control values around twenty-four to forty-eight hours after the injection. The counts of the heterophils drop below the control values during this period. The monocytes follow the pattern of the lymphocytes except that they attain the maximum of overcompensation at later intervals after injection and at higher levels. Due to the infrequency of observation after the first 24 hours it is possible to only tentatively conclude that the period of greatest compensatory increase on the part of the monocytes is in the neighborhood of sixty to seventy hours after the injections, and at levels around 75 per cent of the control values. The monocytes were rather sluggish phagocytically when they began to reappear into circulation, but otherwise no qualitative changes were observed in them. The total counts obviously reflect these opposing events within the component cells, and increase or decrease moderately dependent upon the balance of deviations among the components.

No differences are found between the reactions of the males and females. The reaction of rabbit 49 to subcutaneous injection (Fig. 2) is similar to the reaction following intravenous injection (Fig. 1), except that the reaction of the heterophils in the latter is the more vigorous. The reaction of the heterophils in this particular instance is also more vigorous than the reaction of the heterophils in any of the other rabbits. The reaction of rabbit 49 to subcutaneous injection is entirely comparable to the reactions of the other rabbits to intravenous injection. There seems no question, therefore, of merely a direct toxic action of the intravenously injected hormones upon the cells in circulation.

DISCUSSION

The findings are similar in all respects to those obtained after injections of rabbits with sphingomyelins.[24] The responses of the lymphocytes and heterophils are also similar to their responses following injections of mice, rats and dogs with related extracts or with ACTH.[5, 19] It is now demonstrated from the present data, in addition, that the monocytes also share in the leukopenic action of adrenal cortical extract, and respond to the extract in the same manner as do the lymphocytes.

It is obvious from this finding, therefore, that monocytes and lymphocytes should be weighed individually in assumptions concerning either, as the cytologic locus for the production of specific biologic substances when such assumptions are based merely upon the indirect finding of increments in those substances in the tissues or body fluids secondary to administration of adrenal cortical hormones. This is true whether the substances be immune bodies or other biologic products, and whether elicited by hormones or other experimental procedures. These statements are equally applicable to indirect assumptions concerning the plasma cell as the locus for such production, and are particularly true when forms different from that defined by Marschalkó are under consideration since only that specific morphology is definitive from monocytes in fixed tissues.[3, 4] The so-called "young plasma cells" can be assumed only by indirect reasoning based on transitions.

These data do not necessarily indicate that monocytes are destroyed as a result of the injections of adrenal cortical extract, or that those within the tissues disappear simultaneously with those in the blood. But the parallelism of the reaction of the monocytes to that of the lymyphocytes suggests that the action upon them is similar in both cases. Proof of the disappearance of monocytes within tissues is necessary for certainty, however.

Since monocytes represent young forms of the reticulo-endothelial system on the way to becoming macrophages the question of a possibly similar action of adrenal cortical hormones upon other cells of the system is obvious. The observation of Gordon and Katsh[12] on macrophages and of Dougherty and White[6] on reticular cells of nodes following stimulation of the adrenal cortex are pertinent.

Since monocytes are abundant in not only the blood and lymphoid tissues,[11] but throughout all of the connective tissues,[21] the omentum, mesentery,[20] in most areas of chronic inflammation,[3] and about tubercles, where they become filled with bacilli and transformed into epithelioid cells,[22] this action of adrenal cortical extract upon them is noteworthy.

CONCLUSIONS

1. The response of the circulating white blood cells of rabbits to adrenal cortical extract is identical to their response to sphingomyelins.

2. The monocytes of the blood respond to adrenal cortical extract in the same manner and to the same degree as do the circulating lymphocytes.

3. The findings are discussed as indicative of a need for the differentiation of monocytes from lymphocytes and plasma cells in tissues before conclusions are justified concerning the share of these cells in the production of immune bodies or of other biologic substances.

REFERENCES

1. Bjørneboe, M., and Gormsen, H.: Experimental Studies on the Rôle of Plasma Cells as Antibody Producers, Acta path. et microbiol. Scandinav. **20**: 649, 1943.
2. Bjørneboe, M., Gormsen, H., and Lundquist, F. R.: Further Experimental Studies on the Rôle of the Plasma Cells as Antibody Producers, J. Immunol. **55**: 121, 1947.
3. Bloom, William: The Relationships Between Lymphocytes, Monocytes, and Plasma Cells, Folia hemat. **37**: 63, 1928.
4. Bloom, William: Lymphatic Tissue; Lymphatic Organs, Handbook of Hematology (Hal Downey), New York, 1938, Paul B. Hoeber, Inc., vol. II, p. 1427.
5. Dougherty, Thomas F., and White, Abraham: Influence of Hormones on Lymphoid Tissue Structure and Function. The Rôle of the Pituitary Adrenotrophic Hormone in the Regulation of the Lymphocytes and Other Cellular Elements of the Blood, Endocrinology **35**: 1, 1944.
6. Dougherty, Thomas F., and White, Abraham: Functional Alterations in Lymphoid Tissue Induced by Adrenal Cortical Secretion, Am. J. Anat. **77**: 81, 1945.
7. Dougherty, Thomas F., Chase, Jeanne, and White, Abraham: Pituitary-Adrenal Cortical Control of Antibody Release from Lymphocytes. An Explanation of the Anamnestic Response, Proc. Soc. Exper. Biol. & Med. **58**: 135, 1945.
8. Ehrich, William E., and Harris, T. N.: The Formation of Antibodies in the Popliteal Lymph Node in Rabbits, J. Exper. Med. **76**: 335, 1942.
9. Ehrich, William E., Drabkin, David L., and Forman, Carolyn: Nucleic Acids and the Production of Antibody by Plasma Cells, J. Exper. Med. **90**: 157, 1949.
10. Fagraeus, Astrid: Antibody Production in Relation to the Development of Plasma Cells, Acta med. Scandinav. **130**: Supp. 204, pp. 122, 1948.
11. Forkner, Claude E.: The Origin of Monocytes in Certain Lymph Nodes and Their Genetic Relation to other Connective Tissue Cells, J. Exper. Med. **52**: 385, 1930.
12. Gordon, Albert S., and Katsh, Grace F.: The Relation of the Adrenal Cortex to the Structure and Phagocytic Activity of the Macrophagic System, Ann. New York Acad. Sc. **52**: 1, 1949.
13. Harris, T. N., Grimm, E., Mertens E., and Ehrich, W. E.: The Rôle of the Lymphocyte in Antibody Formation, J. Exper. Med. **81**: 73, 1945.
14. Harris, T. N., and Ehrich, W. E.: The Fate of Injected Particulate Antigens in Relation to the Formation of Antibodies, J. Exper. Med. **84**: 157, 1946.
15. Harris, T. N., and Harris, Susanna: Histochemical Changes in Lymphocytes During the Production of Antibodies in Lymph Nodes of Rabbits, J. Exper. Med. **90**: 169, 1949.
16. Kass, Edward H.: The Occurrence of Normal Serum Gamma-Globulin in Human Lymphocytes, Science **101**: 337, 1945.
17. Keuning, F. J., and Van der Slikke, L. B.: The Rôle of Immature Plasma Cells, Lymphoblasts and Lymphocytes in the Formation of Antibodies as Established in Tissue Culture Experiments, J. Lab. & Clin. Med. **36**: 167, 1950.
18. Metchnikoff, Elei: Immunity in Infective Diseases, Translated by Francis G. Binnie. Cambridge at the University Press. 1907, pp. 591.
19. Reinhardt, William O., Aron, Henry, and Li, Choh Hao: Effect of Adrenocorticotrophic Hormone on Leucocyte Picture of Normal Rats and Dogs, Proc. Soc. Exper. Biol. & Med. **57**: 19, 1944.
20. Sabin, Florence R.: Cellular Reactions to a Dye-Protein with a Concept of the Mechanism of Antibody Formation, J. Exper. Med. **70**: 67, 1939.
21. Sabin, Florence R., Doan, Charles A., and Cunningham, R. S.: Discrimination of Two Types of Phagocytic Cells in the Connective Tissues by the Supravital Technique, Carnegie Inst., Washington, Contributions to Embryology **82**: 125, 1925.
22. Sabin, Florence R., and Forkner, Claude E.: Studies on Tuberculosis, J. Exper. Med. **52**: Supp. 3, 1, 1930.
23. Tompkins, Edna H.: The Nucleinate-Like Action upon the White Blood Cells of the Ether-In-Soluble Fraction of Lipoids from Beef Brains, Bull. Johns Hopkins Hosp. **72**: 347, 1943.
24. Tompkins, Edna H.: Sphingomyelins: Their Action on Blood Cells, Particularly Lymphocytes; Their Share in the Nucleinate-Like Action of the Ether-In-Soluble Fraction of Brain Lipoids, Bull. Johns Hopkins Hosp. **78**: 57, 1946.
25. White, Abraham, and Dougherty, Thomas F.: The Rôle of Lymphocytes in Normal and Immune Globulin Production, and the Mode of Release of Globulin from Lymphocytes, Ann. New York Acad. Sc. **46**: 859, 1946.

RESPONSE OF THE SERUM POLYSACCHARIDE FRACTIONS AND PROTEIN FRACTIONS FOLLOWING CORTISONE TREATMENT OF PATIENTS WITH RHEUMATIC FEVER

M. R. Shetlar, Ph.D., Henry L. Schmidt, Jr., M.D., Richard B. Lincoln, M.D., James K. DeVore, M.D., Jane A. Bullock, M.S., and Arthur A. Hellbaum, M.D., Ph.D.
Oklahoma City, Okla.

THE clinical use of cortisone in the treatment of rheumatic fever has now been the subject of a number of investigations (Barnes,[1] Harris, Abrams, Leo, and Hubbard,[2] and Bell, Bell and Wilson[3]). The mechanism of action of this hormone, however, is still uncertain. Several investigators, including Dorfman and Moses,[4] have suggested that the action of adrenal hormones might be due to their inhibitory effect on connective tissue formation. Layton[5] suggested that this effect is due to repression of synthesis of the polysaccharide moiety of connective tissue. As previous work by one of us has shown that serum polysaccharides are elevated in rheumatoid arthritis,[6] the following study was undertaken to determine whether any relationship might be found between the serum polysaccharides and the clinical response of the rheumatic fever patient to cortisone. At the same time other laboratory studies of possible value in following the clinical course of patients with rheumatic diseases were investigated.

MATERIALS AND METHODS

In a preliminary study, five patients with rheumatic fever were investigated. Two patients with inactive cases, ages 27 and 31 years, were found to have normal serum nonglucosamine polysaccharide levels of 123 and 117 milligrams per 100 milliliters (polysaccharide ÷ protein, 1.91 per cent and 1.70 per cent), and in one early case the patient, aged 17 years, was found to have only a slightly elevated level of 129 (2.11 per cent) ten days after first onset of symptoms. However, in two cases of recurrent rheumatic fever the patients, aged 12 and 29 years, were found to have high polysaccharide levels of 198 and 154 (3.16 per cent and 2.47 per cent).

Five male patients whose ages ranged from 21 to 29 years were then studied in detail. Four had undergone previous episodes of rheumatic fever from one to eleven years prior to the current treatment. One patient could recall no prior rheumatic fever; one had experienced four episodes of recurrent disease; the other three had each experienced one previous episode. None gave history of scarlet fever, "quinsy," or repeated severe sore throats. Two of the patients were farmers, one a clerk, one a chemistry laboratory technician, and one a student in college.

Following a control study period, cortisone acetate was administered intramuscularly. Blood samples were taken every week for the following determinations: total nonglucosamine polysaccharide, glucosamine polysaccharide, albumin polysaccharide, α-globulin

From the Medical Service and the Research Laboratory, Veterans Administration Hospital, and the Oklahoma Medical Research Foundation.

Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

This is publication No. 15 from the Oklahoma Medical Research Institute.

Received for publication, Aug. 27, 1951.

polysaccharide, hexosamine, serum protein, albumin, α-globulin, sedimentation rate, blood glucose, the heat coagulation test of Huggins and associates,[7] serum sodium, and serum potassium.

Total nonglucosamine serum polysaccharide, glucosamine polysaccharide, albumin polysaccharide, α-globulin polysaccharide, total serum protein, albumin and α-globulin were determined as previously described by Shetlar and co-workers.[8, 9] Serum and urinary sodium and potassium were determined by flame photometry using a Jenke Flame Photometer as described by Fox.[10] Urinary corticoid substances were determined by a method modified from that of Talbot and associates.[11]

RESULTS

In all cases initially high serum nonglucosamine polysaccharide, glucosamine polysaccharide, and albumin polysaccharide levels were found as compared to the normal values previously determined.[8] In all five cases elevated α-globulin polysaccharide levels were found when the polysaccharide was expressed in milligrams per 100 cubic centimeters of serum, but the α-globulin polysaccharide expressed as a percentage of the α-globulin was normal in one case (C. A. I.). This patient had been treated with salicylates for twenty-four days before the first blood sample was taken and before cortisone therapy was started. Although joint symptoms had recurred, the patient's temperature was normal at this time. A correlation between elevation of the α-globulin polysaccharide expressed as a percentage of the α-globulin and temperature elevation is indicated. Animal work confirming this statement will be published elsewhere.[13] Low serum albumin percentage, low albumin/globulin ratio, and high serum heat coagulation figures were also found for all cases. Initial results together with normal values are tabulated in Table I.

In general, the changes of the nonglucosamine, glucosamine, serum albumin and α-globulin polysaccharide levels closely paralleled subjective improve-

TABLE I. INITIAL SERUM VALUES OF RHEUMATIC FEVER PATIENTS AS COMPARED TO NORMAL VALUES

	NORMAL AVERAGE	L.D.W.	C.A.I.	C.E.S.	E.G.A.	R.E.R.
Nonglucosamine serum polysaccharide*	121 (93-126)	259	204	271	220	280
Nonglucosamine serum polysaccharide ÷ serum protein†	1.58 (1.26-2.02)	3.77	2.87	3.51	2.91	3.87
Glucosamine polysaccharide*	67 (61-78)	178	129	151	143	194
Glucosamine polysaccharide ÷ serum protein†	0.96 (0.84-1.26)	2.60	1.81	1.96	2.05	2.68
Albumin polysaccharide*	29 (26-37)	102	45	64	55	49
Albumin polysaccharide ÷ albumin†	0.70 (0.61-0.82)	3.03	1.43	1.99	1.63	1.52
α-Globulin polysaccharide*	19 (14-23)	37	41	67	67	72
α-Globulin polysaccharide ÷ α-globulin†	2.80 (1.89-4.00)	4.25	3.28	5.11	5.08	5.14
Heat coagulation test‡	1.18 (1.08-1.28)	1.72	1.64	1.90	1.86	2.02
Albumin percentage§	62 (53-70)	50	44	42	48	45

*Expressed as milligrams per cent.
†Expressed as a percentage of the respective protein moiety.
‡Expressed as the lowest coagulable protein concentration.
§Expressed as a percentage of the total serum protein.

ment and the temperature charts (Figs. 1, 3, and 4). The decrease of these factors appeared to lag behind favorable clinical response by a matter of a few days. Although a rapid decrease of the nonglucosamine, glucosamine, and albumin polysaccharide occurred soon after the patients became asymptomatic, the appearance of normal values did not occur for some time after continued therapy (Table II). The heat coagulation test values of all patients decreased

TABLE II. DAYS AFTER BEGINNING OF THERAPY REQUIRED FOR FIRST APPEARANCE OF NORMAL VALUES

FACTOR	L.D.W.	C.A.L.	C.E.S.	E.G.A.	R.E.R.	AVERAGE
Serum polysaccharide	71	55	33	54	56	55
Albumin polysaccharide	92	41	33	33	35	47
α-globulin polysaccharide	26	0	27	40	13	21
Heat coagulation test*	13	28	27	47	42	31
A/G ratio	0	0	20	0	0	--
Sedimentation rate†	11	41	*	36	*	--
Fever	5	2	3	17	5	6
Objective joint findings‡	3	0	4	8	6	4
Subjective joint symptoms§	4	2	2	6	23	5

*Determined by Howe method.

†Sedimentation rate failed to become normal at any time.

‡Presence of swelling, redness, or local increase in temperature in the joints, or joint effusion.

§Any complaint of joint pain by the patient upon movement of joint or any subjective sensation of joint pain.

to normal under treatment, but did not seem to be as closely correlated with clinical observations as the serum and albumin polysaccharide. Albumin expressed as a percentage of the total protein and the albumin/globulin ratio (albumin ÷ α-, β-, σ-globulin) as determined by the Milne method[12] seemed closely correlated with clinical changes. The albumin/globulin ratio determined by the Howe method (albumin ÷ α-globulin ÷ β- and σ-globulin) was a much poorer indication of patient status. The erythrocytes sedimentation rate decreased and showed some relation to clinical changes in three cases, but remained elevated in two cases, although these cases improved clinically. No significant changes were noted in serum sodium, serum potassium, urinary sodium, and urinary potassium. The urinary corticoid substances appeared to follow the level of cortisone therapy in a general way, increasing when cortisone was given, and falling to levels lower than the original ones when the therapy was discontinued.

ILLUSTRATIVE CASES

CASE 1.—Patient L. D. W., a 27-year-old white man, was first hospitalized with history of four days' duration of fever and migratory joint symptoms consisting of pain and swelling without redness. The patient was acutely ill with a temperature of 102° F.; pulse, 100; and respiration, 60. Fine moist râles were heard posteriorly over the lower two-thirds of both lungs, and a loud friction rub over the entire precordium was noted. A roentgenogram of the thorax revealed a massively increased cardiac density with a globular configuration. The electrocardiogram showed a rate of 100 with a P-R interval of 0.20 second. The white blood count was 11,800 per cubic millimeter, and the sedimentation rate was 36 millimeters per hour. The nonglucosamine polysaccharide was 259 mg. per cent; glucosamine polysaccharide, 178 mg. per cent; albumin polysaccharide, 102 mg. per cent; α-globulin polysaccharide, 37 mg. per cent; albumin percentage, 50; heat coagulation test value, 1.72; serum sodium, 290 mg. per cent; serum potassium, 16 mg. per cent; and urinary corticoids, 1.46 mg. per day.

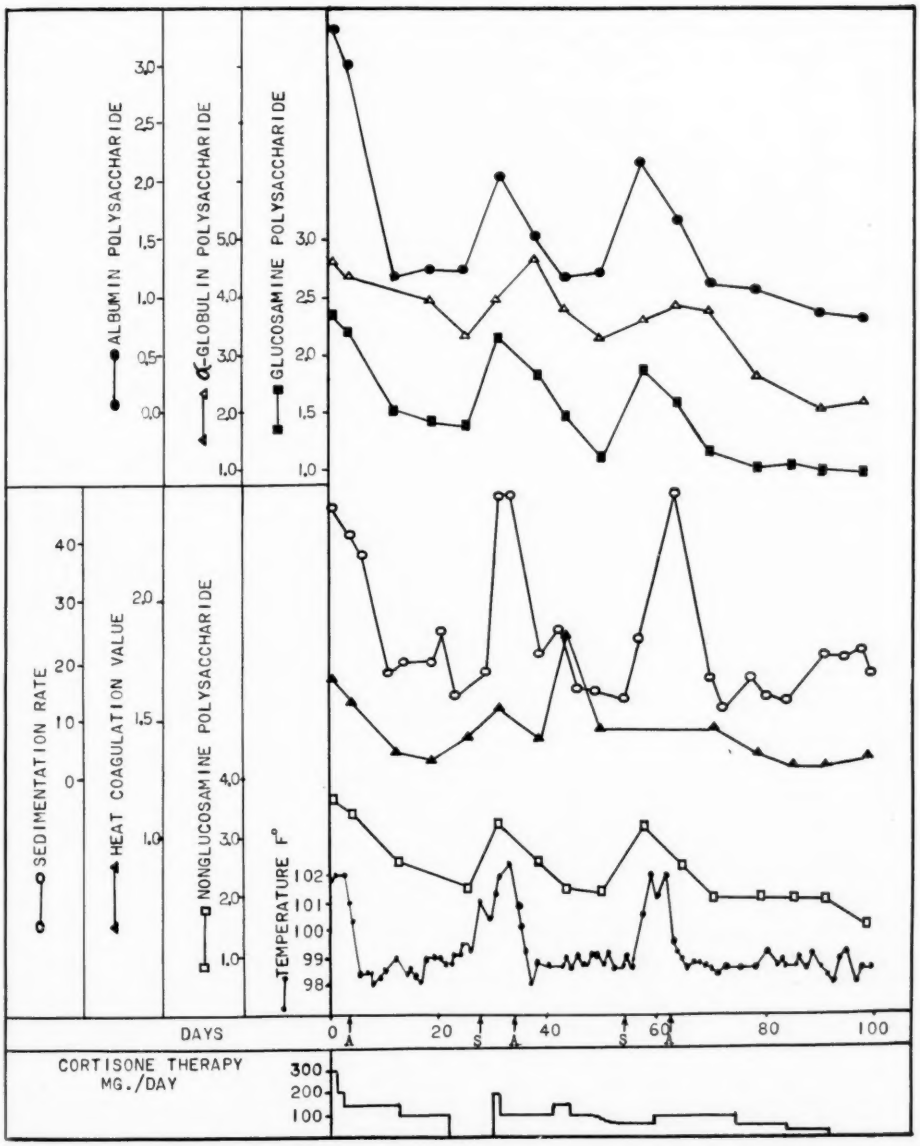


Fig. 1.—Patient L. D. W. Response of the total nonglucosamine polysaccharide, heat coagulation value, sedimentation rate, glucosamine polysaccharide, albumin polysaccharide, and α-globulin polysaccharide following cortisone treatment. The nonglucosamine and glucosamine polysaccharides are expressed as percentages of the total serum protein; albumin and α-globulin polysaccharides are expressed as percentages of the serum albumin and α-globulin, respectively. The sedimentation rate is expressed in millimeters per hour, and the heat coagulation value is expressed as the least coagulable protein concentration, i.e., the least coagulable percentage of serum multiplied by the grams of total protein in 100 cubic centimeters of serum. The level of cortisone therapy in milligrams per day and the temperature chart are also shown for comparison with laboratory data.

The patient first became asymptomatic on the third day after starting therapy. This day is indicated by A↑. A drop in glucosamine, nonglucosamine, albumin, and α-globulin polysaccharide levels, heat, coagulation value, and sedimentation rate was noted on the fifth day. After stopping treatment on the twenty-first day, joint symptoms recurred the twenty-ninth day, which is indicated by S↑. An elevation of the polysaccharide components, sedimentation rate and heat coagulation value was noted soon after on the thirty-second day. Note that the α-globulin polysaccharide lags behind the albumin polysaccharide in response. A second recurrence of joint symptoms occurred on the fifty-fifth day.

On the third hospital day cortisone was started with a dose schedule as follows: 300 mg. first day, 200 mg. second day, and 150 mg. daily for ten days and 100 mg. daily for ten days.

Two days after starting therapy the patient became asymptomatic. As shown in Figs. 1 and 2, the serum nonglucosamine and glucosamine polysaccharide, the albumin polysaccharide, the sedimentation rate, and the α-globulin percentage all fell to lower levels by the fifth day after starting therapy. The albumin percentage and albumin/globulin ratios were slower in responding, but rose by the thirteenth day. Urinary corticoids were found to be further elevated to 3.51 mg. per day by the tenth day.

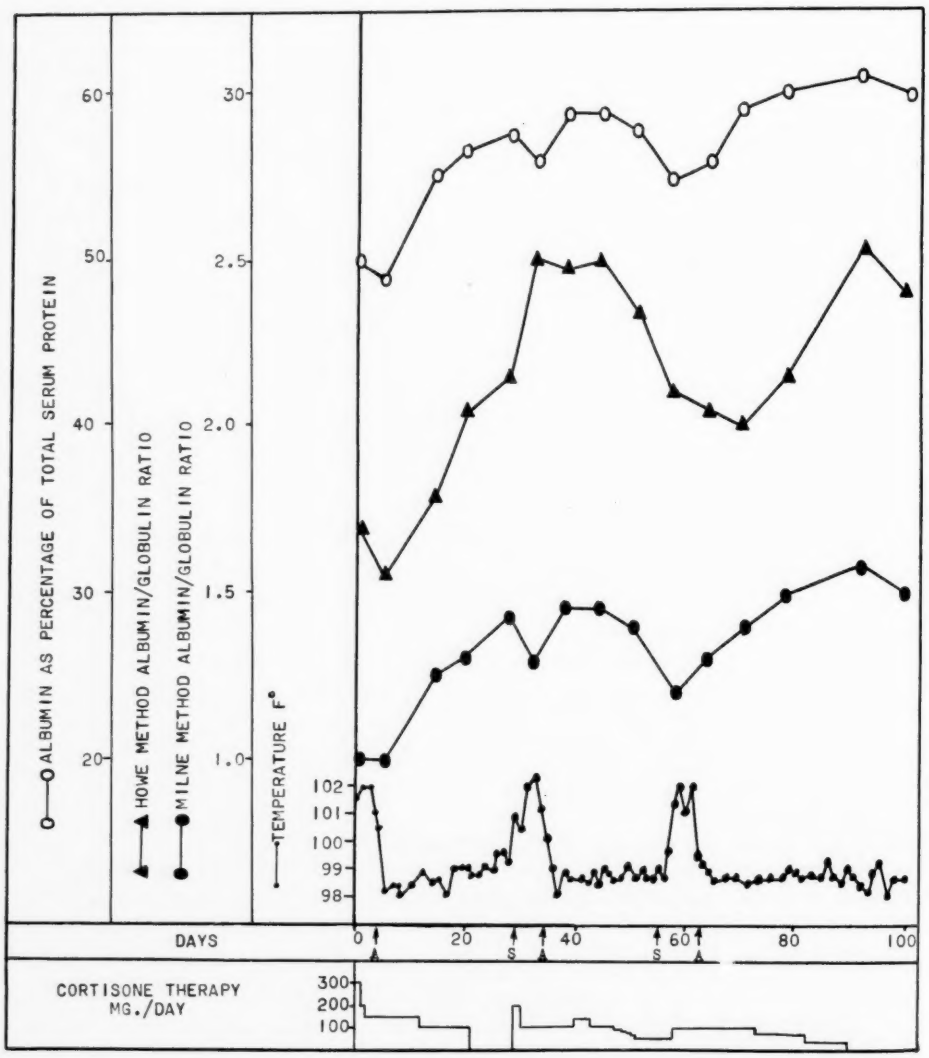


Fig. 2.—Patient L. D. W. Response of the albumin percentage, albumin/globulin ratio determined by the Milne method, and albumin/globulin ratio determined by the Howe method to cortisone treatment. Note that following treatment with cortisone and clinical improvement of the patient, the albumin percentage (serum albumin per 100 grams of total serum protein) and albumin/globulin ratios increased. Following recurrence of joint symptoms (indicated by S↑) the albumin percentage and albumin/globulin ratio determined by the Milne method fell. A poorer relationship of clinical symptoms to the albumin/globulin ratio determined by Howe's method is noted.

Medication was discontinued after the twenty-second day of therapy. Three days later there was a recurrence of fever and tachycardia and in two more days chest pain and orthopnea were noted. On the tenth day after cessation of therapy blood samples were taken and the total, albumin and α-globulin polysaccharide levels, the sedimentation rate, and α-globulin all rose again, while the albumin percentage and Milne albumin/globulin ratio fell. The Howe albumin/globulin ratio failed to respond in this instance due to the concurrent increase in α-globulin. Cortisone medication was resumed on the fifth day after cessation of the original course of therapy with an initial dose of 200 mg. and a daily dose of 100 mg. for ten days. The dose was then reduced by 10 mg. daily until a level of 50 mg. per day was reached. This level was maintained for seven days. On the sixth day at the 50 mg. level, an increase in total serum and albumin polysaccharide and a decrease in albumin percentage and albumin/globulin was noted. The next day fever and tachycardia again recurred and the dose was increased to a 100 mg. level. Symptoms subsided slowly at this level of therapy and the patient became asymptomatic after five days. This was quickly followed by a corresponding drop in serum nonglucosamine polysaccharide, glucosamine polysaccharide, albumin polysaccharide, and an increase in albumin percentage and albumin/globulin ratio by the Milne procedure. The α-globulin polysaccharide continued to rise until nine days after the patient became asymptomatic when a slight drop was noted.

After fifteen days at the 100 mg. level, the dose was reduced to 75 mg. daily and reduced further to 25 mg. daily nine days later. Therapy was discontinued after eight days at this level. Physical examination disclosed the heart to be entirely normal with a blood pressure of 128/90. A roentgenogram of the thorax was reported as being within normal limits. During the period of this study a series of twenty-eight electrocardiograms showed transitory ST segment elevations and T-wave inversions. The last ten electrocardiograms, however, were considered within normal limits.

CASE 2.—Patient C. A. L., a 21-year-old white man, first had symptoms in 1941, followed by hospitalization on four occasions because of typical acute symptomatology. Findings consistent with both aortic and mitral valvular damage had been observed repeatedly. Ten days prior to admission he developed an upper respiratory infection associated with a sore throat and five days later noticed fever, increased malaise, and migratory joint symptomatology. Upon admission the patient appeared acutely ill with a temperature of 98.6 F.; pulse, 110; respiration, 20; blood pressure, 100/60. Tachycardia and a precordial thrill were noted. The right ankle showed questionable swelling, and the shoulder joints were painful on motion. Laboratory findings were: red blood count, 3,720,000 per cubic millimeter; hemoglobin, 11.9 Gm.; white blood count, 15,200 per cubic millimeter; sedimentation rate, 38 mm. per hour. A roentgenogram of the thorax showed no obvious heart enlargement, but was suggestive of "mitral" configuration. The electrocardiogram showed a rate of 90, a P-R interval of 0.26 second, and changes suggestive of left ventricle hypertrophy.

After admission the patient was treated with bed rest and salicylates, 6 Gm. daily, and gradually became less symptomatic. On the twenty-first day of salicylate therapy temperature became elevated, the P-R interval lengthened from 0.18 to 0.22 second, and the left first metacarpophalangeal and right knee joints became swollen, reddened, hot, and tender.

Laboratory studies on the twenty-fourth hospital day revealed results similar to those described in the previous case with a sedimentation rate of 40 mm. per hour, high total, albumin and α-globulin polysaccharide levels, high serum heat coagulation test value with lowered albumin percentage as shown in Table I.

On the twenty-fourth hospital day cortisone medication was instituted with a daily dose schedule as follows: 300 mg. first day, 200 mg. second day, and 100 mg. for twelve days, at which time cortisone therapy was discontinued. Symptomatic improvement was dramatic and the polysaccharide and other serum factors responded (Fig. 3) as described in the previous case.

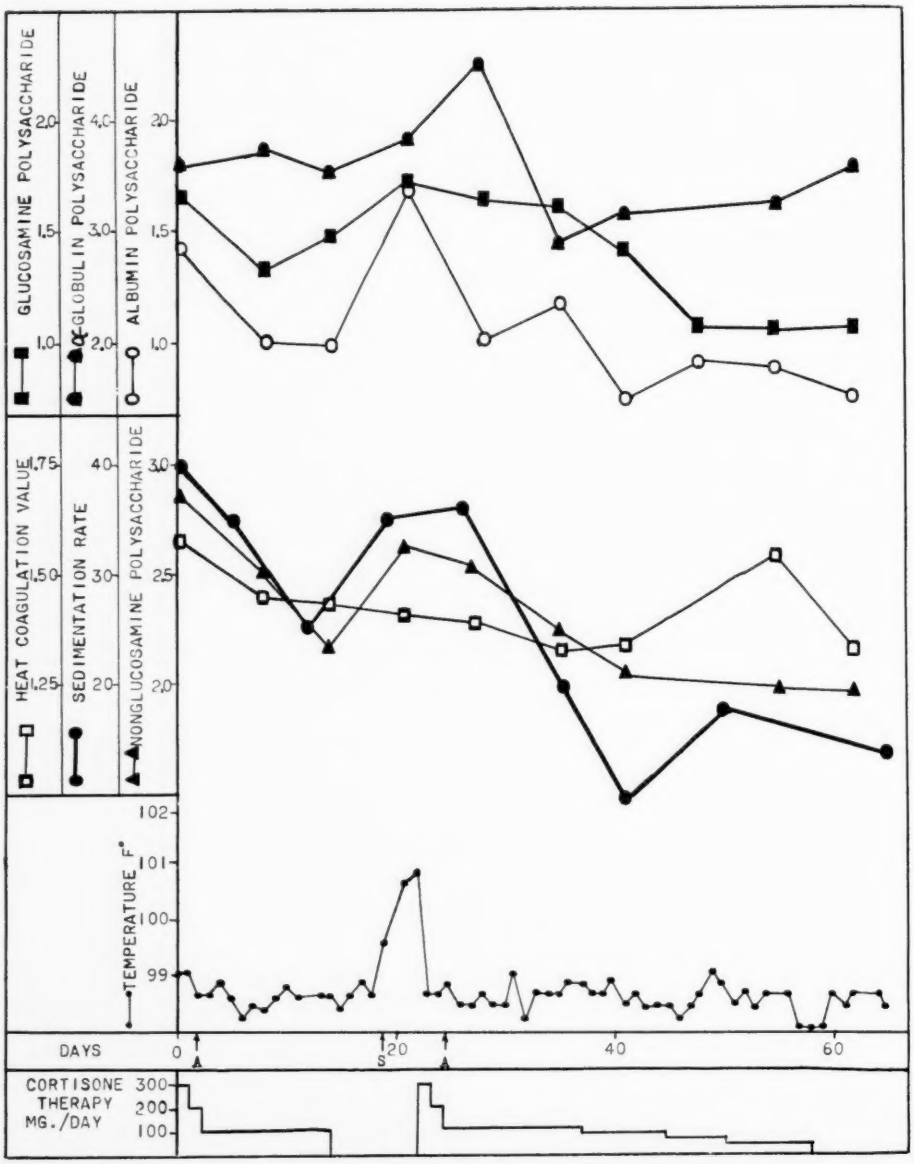


Fig. 3.—Patient C. A. I. Response of the total nonglucosamine polysaccharide, heat coagulation value, sedimentation rate, glucosamine polysaccharide, albumin polysaccharide and α-globulin polysaccharide following cortisone treatment.

The nonglucosamine and glucosamine polysaccharides are expressed as percentages of the total serum protein; albumin and α-globulin polysaccharides are expressed as percentages of the serum albumin and α-globulin, respectively. The sedimentation rate is expressed in millimeters per hour and the heat coagulation value is expressed as the least coagulable protein concentration, i.e., the least coagulable percentage of serum multiplied by the grams of protein in 100 cubic centimeters of serum. The level of cortisone therapy in milligrams per day and the temperature chart are also shown for comparison with the laboratory data. The patient first became asymptomatic on the second day after starting therapy. This day is indicated by A↑. A drop in glucosamine, nonglucosamine, and albumin polysaccharide levels, heat coagulation value and sedimentation rate was noted on the eighth day. After stopping treatment on the fourteenth day, joint symptoms recurred on the nineteenth day. This day is indicated by S↑. An elevation of the polysaccharide components and sedimentation rate was noted on the twenty-first day, but the heat coagulation value continued to drop. Upon resumption of cortisone therapy the patient became asymptomatic on the twenty-fifth day. This was followed by a drop in the polysaccharide components and sedimentation rate.

Four days after cessation of cortisone therapy an increased temperature and pulse rate were noted and the patient again complained of joint pain without objective findings. This recurrence was followed by a striking change in laboratory results as depicted in Fig. 3. Cortisone therapy was resumed three days later with the following daily dosage: 300 mg. first day, 200 mg. second day, and 100 mg. daily thereafter for thirteen days, 75

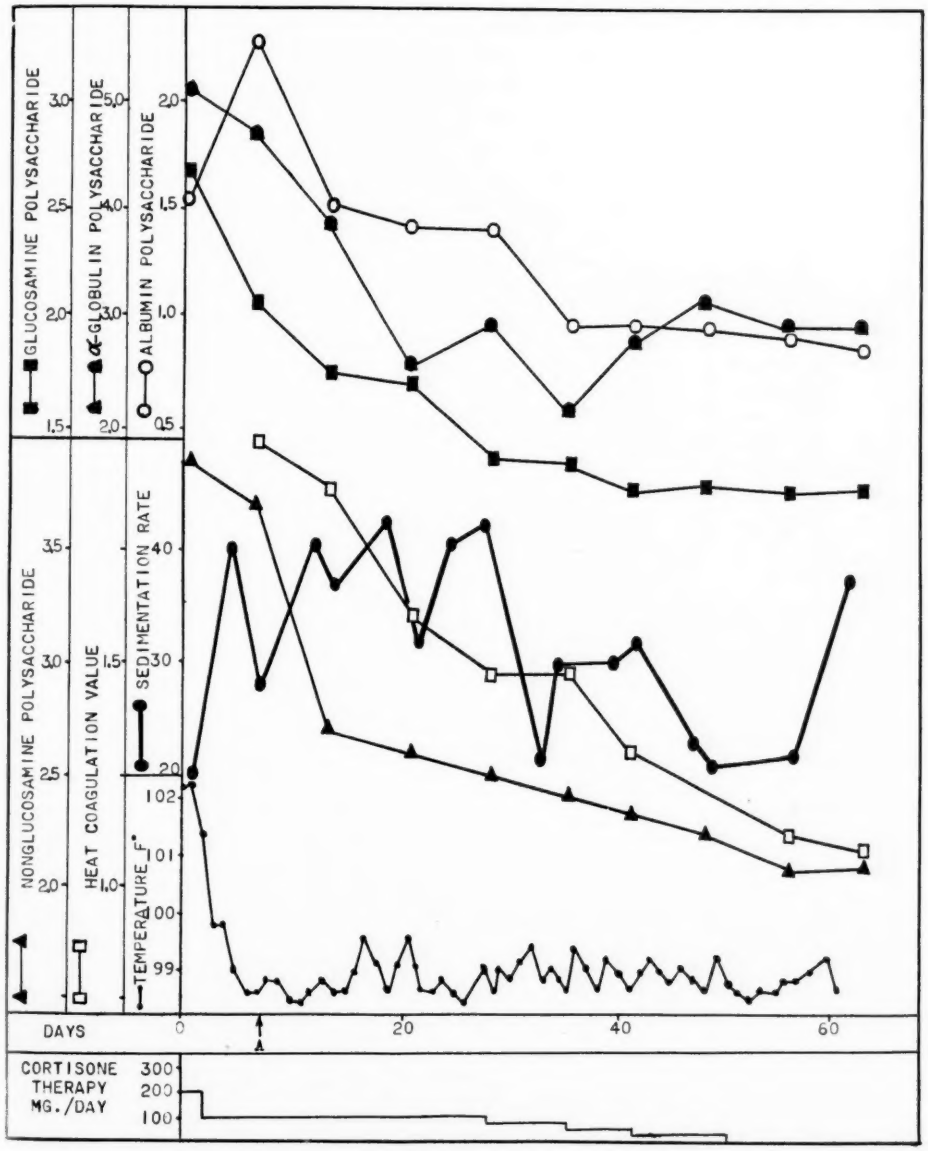


Fig. 4.—Patient R. F. R. Response of the total nonglucosamine polysaccharide, heat coagulation value, sedimentation rate, glucosamine polysaccharide, albumin polysaccharide and α-globulin polysaccharide following cortisone treatment. This patient responded clinically to cortisone treatment much less rapidly than did other patients. This slower response is perhaps correlated with a continued rise in albumin polysaccharide the first week. Note that the α-globulin polysaccharide fell during this period. On the fifteenth day, the polysaccharide components and the heat coagulation value had all decreased. The sedimentation rate, however, remained elevated throughout the course of the treatment.

mg. daily for seven days, 50 mg. daily for seven days, and 25 mg. daily for seven days. Symptomatic improvement was again noted to be prompt. The patient became afebrile within twenty-four hours and his tachycardia rapidly disappeared.

Laboratory results again reflected this improvement (Fig. 3). After the final discontinuation of cortisone there were no further exacerbations of symptoms. The urinary corticoids fell to a level of 0.35 mg. per day six days after cessation of cortisone. The physical examination of the heart showed the positive findings as previously described. A roentgenogram of the thorax was reported as showing no change. A series of nineteen electrocardiograms recorded during this period showed a P-R interval which varied between 0.16 and 0.26 second. Transitory T-wave changes in amplitude were observed. The changes suggestive of left ventricle hypertrophy remained constant.

CASE 3.—Patient R. F. R., a 28-year-old white man, was hospitalized complaining of malaise, fever, and diffuse joint pains of two weeks' duration. One month prior to admission the patient had noted a sore throat. A week following onset of joint symptoms and a week prior to admission the patient had received salicylates and penicillin, and on admission had a moderately severe urticaria, thought to be a complication of the penicillin medication, which made the evaluation of his atypical generalized and progressive joint symptomatology a difficult problem. Physical examination showed the patient to be well developed and nourished, but acutely ill. Temperature was 101.2° F.; pulse, 88; respiration, 20. A catarrhal otitis media, left, was noted. The cardiac examination showed no abnormalities. The left knee, left ankle, and right elbow were swollen, hot, and tender without redness. Laboratory findings were: white blood count, 11,100 per cubic millimeter; high total, albumin and α-globulin polysaccharides, high serum heat coagulation test values, low albumin percentage and albumin/globulin ratio, sedimentation rate of 18 mm. per hour. A roentgenogram of the thorax was reported as showing a platelike atelectasis in the left lung base. The electrocardiogram revealed a rate of 102, with a P-R interval of 0.23 second and inversion of the T waves in Leads V_5 and V_6.

After admission, the patient received 200 mg. daily of cortisone for the first two days. Thereafter he received a daily dosage of 100 mg. daily for twenty-six days, 75 mg. daily for seven days, 50 mg. daily for six days, and 25 mg. daily for nine days. The patient became afebrile seventy-two hours after the institution of therapy. His joint symptoms subsided much less dramatically than did those of previous patients, but the sixth day after starting therapy he was relatively asymptomatic, although he continued to have vague complaints in regard to various joints without objective findings.

Laboratory studies reflected the slower clinical response of this patient to therapy (Fig. 4). The total serum nonglucosamine polysaccharide level dropped only slightly by the seventh day, while the albumin polysaccharide increased, and the albumin percentage and albumin/globulin ratios decreased during the same period. On the other hand the α-globulin polysaccharide decreased as rapidly as in the previous cases. Shetlar, Knobloch, Shetlar, Richmond, and Everett[13] noted a close correlation of the rise of this factor with fever, consequently the decrease of α-globulin polysaccharide may be connected in this case with the drop in temperature, while the rise in albumin polysaccharide may indicate the persistance of a proliferative process. The sedimentation rate continued elevated, with an average corrected reading of 30 mm. per hour. A roentgenogram of the thorax was reported entirely normal. A series of eighteen electrocardiograms recorded during hospitalization showed an abnormally prolonged P-R interval until the thirteenth day after start of cortisone therapy and transitory T-wave negativity for six days longer. The last nine electrocardiograms were reported as being within normal limits.

DISCUSSION

Data presented here indicate a close correlation between serum polysaccharide factors, and the clinical improvement of rheumatic fever patients under cortisone therapy. Whenever the patients improved clinically, the polysac-

charide components decreased. Conversely, when symptoms recurred these components rose again. At the present time, it is not possible to ascribe the effects obtained definitely to recovery from disease or to administration of cortisone itself. However, administration of cortisone to a few normal dogs failed to alter materially any of the polysaccharide components. Work to clarify this question is currently under way. Previous evidence that the albumin polysaccharide may be correlated with tissue proliferation[14] and the findings of Plotz, Howes, Meyer, Blunt, Lattes, and Ragan,[15] Spain, Molomut, and Haber,[16] and Taubenhaus and Amromin,[17] indicating that cortisone will inhibit connective tissue formation are indeed suggestive. The discovery of Layton[5] that cortisone will inhibit the synthesis of chrondroitin sulfuric acid in vitro leads one to the interesting postulation that the effect of cortisone on joint symptoms may be due to its inhibition of chrondroitin sulfate synthesis and the consequent inhibition of connective tissue proliferation. The increase of albumin polysaccharide may be a reflection of the increased formation of connective tissue in rheumatic fever.

An elevation of α-globulin polysaccharide has been shown to occur in experimental animals whenever a temperature elevation occurs.[13] Some of the elevation found in rheumatic fever is doubtlessly due to the fever which occurs in the disease. This was further indicated by the fact that the α-globulin polysaccharide fell in two cases (for example, Fig. 4), in whom fever fell after the administration of cortisone, but in whom joint symptoms persisted for some time and in whom the albumin polysaccharide level became more elevated after the start of therapy.

The apparently close correlation of the polysaccharide levels and the heat coagulation test may not necessarily be fortuitous as the presence of abnormal amounts of polysaccharide-rich serum protein probably inhibits coagulation of protein by heat. Seromucoid is not heat coagulable and similar carbohydrate proteins may have the same property. Likewise, the close correlation which was found in some cases between polysaccharide levels and sedimentation rate may not be purely coincidental as Meyer, Hahnel, and Feiner[18] have shown that hyaluronic acid will cause a great increase in sedimentation rate in vitro and in vivo. It is possible that other polysaccharides may also have this property. Needless to say, other factors also influence the sedimentation rate.

Changes in the albumin percentage as determined by the Milne method[12] seem to be correlated with subjective improvement of the rheumatic fever patient. As the albumin fractionated by the Howe method contains most of the α-globulin, the albumin/globulin ratio obtained by the Howe method is a much poorer indication of the status of the patient. The Milne method might be preferable to the Howe method for studies on rheumatic fever cases.

SUMMARY

A preliminary study was made of alterations of the serum polysaccharides and other serum factors occurring in five rheumatic fever patients under treatment with cortisone. The serum nonglucosamine polysaccharide, glucosamine polysaccharide, albumin polysaccharide, α-globulin polysaccharide and serum

heat coagulation values were all found to be greatly elevated prior to therapy, while the albumin percentage and albumin/globulin ratios were lowered. Following treatment with cortisone, subjective clinical improvement was promptly followed by decreases in the serum nonglucosamine and glucosamine polysaccharides, albumin polysaccharide, α-globulin polysaccharide and serum heat coagulation values and by increases in albumin and in the albumin/globulin ratio. Recurrence of rheumatic fever after cessation of treatment occurred in two cases and was accompanied by a return of the serum factors to the original state.

It appears that the studies of the serum polysaccharide components, serum heat coagulation values and albumin percentages may be valuable aids in the evaluation of rheumatic fever patients.

REFERENCES

1. Barnes, A. R.: Effects of Cortisone and ACTH in 14 Patients With Acute Rheumatic Fever, Proc. Staff Meet., Mayo Clin. **25**: 478-479, 1950.
2. Harris, T. N., Abrams, W. B., Leo, T. F. P., and Hubbard, J. P.: Preliminary Observations on the Effects of Cortisone in the Treatment of Acute Rheumatic Carditis, Am. J. Dis. Child. **80**: 884-885, 1950.
3. Bell, G. I., Bell, R. E., and Wilson, D. R.: Rheumatic Pancarditis Treated With Cortisone, Canad. M. A. J. **63**: 63-66, 1950.
4. Dorfman, A., and Moses, F. E.: Effect of Adrenocorticotrophic Hormone (ACTH) on Serum Hyaluronidase Inhibitor in Rheumatic Fever, Proc. Soc. Exper. Biol. & Med. **73**: 667-669, 1950.
5. Layton, L. L.: Effect of Cortisone Upon Chondroitin Sulfate Synthesis by Animal Tissues, Proc. Soc. Exper. Biol. & Med. **76**: 596-598, 1951.
6. Shetlar, M. R., Shetlar, C. L., Richmond, V., and Everett, M. R.: The Polysaccharide Content of Serum Fractions in Carcinoma, Arthritis, and Infections, Cancer Research **10**: 681-683, 1950.
7. Huggins, C., Cleveland, A. S., and Jensen, E. V.: Thermal Coagulation of Serum in Diagnosis, J. A. M. A. **143**: 11-15, 1950.
8. Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R.: Serum Polysaccharide Level in the Normal State, Proc. Soc. Exper. Biol. & Med. **69**: 507-511, 1948.
9. Shetlar, M. R., Foster, J. V., and Everett, M. R.: Determination of Serum Polysaccharides by the Tryptophane Reaction, Proc. Soc. Exper. Biol. & Med. **67**: 125-130, 1948.
10. Fox, C. L.: Stable Internal Standard Flame Photometer for Potassium and Sodium Analysis, Analytical Chemistry **23**: 137-142, 1951.
11. Talbot, N. B., Saltzman, A. H., Wixom, R. L., and Wolfe, J. K.: The Colorimetric Assay of Urinary Corticosteroid-like Substances, J. Biol. Chem. **160**: 535-547, 1945.
12. Milne, J.: Serum Protein Fractionation: A Comparison of Sodium Sulfate Precipitation and Electrophoresis, J. Biol. Chem. **169**: 595-599, 1947.
13. Shetlar, M. R., Knobloch, W. H., Shetlar, C. L., Richmond, V., and Everett, M. B.: Effect of Fever on Serum Polysaccharides, In preparation.
14. Shetlar, M. R.: The Polysaccharides of Serum Proteins in Cancer, Texas Reports on Biol. & Med., In press, March, 1952.
15. Plotz, C. M., Howes, E. L., Meyer, K., Blunt, J. W., Lattes, R., and Ragan, C.: The Effect of the Hyperadrenal State on Connective Tissue, Am. J. Path. **26**: 709-710, 1950.
16. Spain, D. M., Molomut, N., and Haber, A.: The Effect of Cortisone on the Formation of Granulation Tissue in Mice, Am. J. Path. **26**: 710-711, 1950.
17. Taubenhaus, M., and Amromin, G. D.: The Effects of the Hypophysis, Thyroid, Sex Steroids, and the Adrenal Cortex Upon Granulation Tissue, J. Lab. & Clin. Med. **36**: 7-18, 1950.
18. Meyer, K., Hahnel, E., and Feiner, R.: Experiments on Erythrocyte Sedimentation Rate, Proc. Soc. Exper. Biol. & Med. **58**: 36-40, 1945.

STUDIES OF THE CORTISONE EFFECTS ON THE INFLAMMATORY RESPONSE

I. Alterations of the Histopathology of Chemically Induced Inflammation

David M. Spain, M.D., Norman Molomut, Ph.D., and Alvin Haber, B.S.
Valhalla, N. Y., and Brooklyn, N. Y.

THE widespread therapeutic use of cortisone and ACTH, with their beneficial effects on specific conditions such as rheumatoid arthritis has led to instances of adverse effects on patients attending their use. Some such adverse effects are: activation of peptic ulcer;[1, 2] perforation of intestine;[3] unexpected rapid decubitus ulcer development;[4] masked peritonitis;[5] delay in abscess healing;[6] and the development or enhancement of active pulmonary tuberculosis.[7-9] In various experimental animals cortisone and/or ACTH have altered the host's response to allergic,[10] bacterial,[11] viral,[12] and chemical agents.[13] These are all to some extent manifestations of both altered inflammatory and repair mechanisms. It is the purpose of this study to investigate alterations produced by cortisone in the histopathology of the inflammatory responses to further elucidate the mechanisms involved in the prior sited adverse effects.

MATERIALS AND METHODS

Mice used were of the Swiss albino strain, equally divided as to sex, approximately three months of age, weighing 20 to 25 Gm., and controlled for freedom from enteric, respiratory, and Bartonella infection. A standard diet of vitamin and mineral fortified grains in the form of checkers was med ad libitum, as was water. After turpentine injection each mouse was housed individually to avoid the possibility of injury to injection site. At sacrifice the entire suprasternal area was excised, including the muscle layers and formalin fixed. Representative sections were cut and routinely stained with hematoxylin and eosin and fibrin stain, where indicated. A minimum of three sections were made of each inflammation site.

In order to determine the optimal turpentine dose, three groups of twenty mice each were injected subcutaneously over the sternum with 0.1 ml., 0.05 ml., and 0.025 ml., respectively, of steam-distilled turpentine. Animals from each group were sacrificed at three, six, twelve hours, and one, two, three, four, five, six, seven, and fourteen days. Following histologic examination of the above inflammation sites it was decided that the optimal dose was 0.025 ml. The procedure as outlined in Table I was then performed (see Table I).

The effect of cortisone pretreatment on the response to 0.1 ml. of turpentine was determined on five mice pretreated with 1 mg. of cortisone daily for three days. These mice were then sacrificed twenty-four hours following 0.1 mg. turpentine injection, and the inflammation sites were sectioned.

The reaction of the exudate was determined by injecting ten mice with 0.025 ml. turpentine at multiple sites over the sternum. At the end of twenty-four hours, three days, and six days, these inflammation sites were aspirated and the pH determined.

From the Department of Laboratories and Research, Westchester County, Valhalla, N. Y., and the Waldemar Medical Research Foundation, Brooklyn, N. Y.

This investigation was supported by a research grant from the National Institute of Health, Public Health Service. (Grant #GG 2979-Path.)

Received for publication, Oct. 15, 1951.

RESULTS

In the Group 2 mice the following was noted as compared with Group 1 (see Table I). In the three-, six-, and twelve-hour period: the edema was less intense and less well localized; the leukocytic zone (both polymorphonuclear leukocytes and mononuclear cells) was scant and practically nil until the twelve-hour sections; margination was delayed and scant even at the twelfth hour; capillary hyperemia was almost entirely absent; fibrin was scant (see Figs. 1 and 2).

In the one-, two-, three-, four-, and five-day sections, the same general suppression of all elements of the inflammatory process continued. Whereas the leukocytic zone in the Group 1 mice was dense, well formed, and complete, in the Group 2 mice it was sparse, poorly defined, and incomplete, with areas of

Fig. 1.

Fig. 2.

Fig. 1.—Twelve-hour control inflammation site with a prominent leukocytic zone (*a*), capillary hypermia, margination (b), fibrin (c), and edema. (H&E ×350.)

Fig. 2.—Twelve-hour cortisone-treated inflammation site with a sparse and incomplete leukocytic zone (*a*), insignificant margination, and scant hyperemia (*b*). In the center between the leukocytic zones is a small amount of fibrin (*c*). (H&E ×350.)

complete absence. In the Group 2 mice fibrin remained scant. The perileukocytic zone in Group 1 contained considerable fibrin, numerous mononuclear cells, and prominent fibroblastic proliferation, while the Group 2 treated animals showed almost no fibrin in the perileukocytic zone, with occasional mononuclear cells and an insignificant degree of fibroblastic proliferation. Necrosis was prominent in the leukocytic zone of the Group 1 and negligible in the Group 2 mice (see Figs. 3 and 4).

In the seventh day the zones of inflammation in Group 1 were composed of well-defined abscesses, with intact and well-formed granulation tissue capsules. Considerable liquefaction necrosis of the cells in the exudate was present. The lesions were well defined from the adjacent muscle and only occasional focal collections of leukocytes were present in the muscle layer. In

Fig. 3.

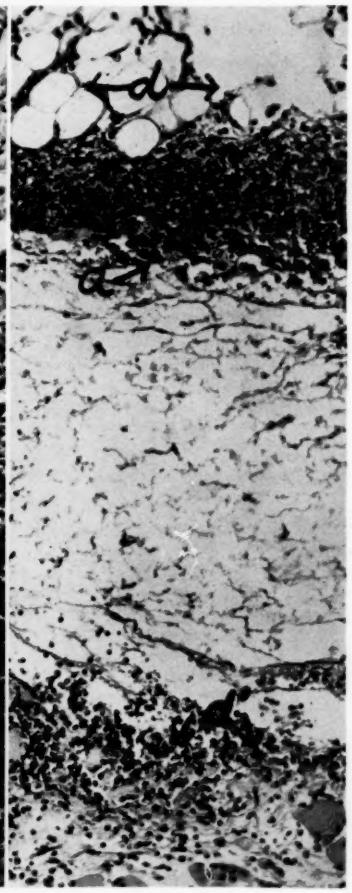


Fig. 4.

Fig. 3.—Forty-eight hour control inflammation site with a dense leukocytic zone with liquefaction necrosis at its inner aspects (*a*). At the periphery there are mononuclear cells and some fibroblasts (*d*). (H&E ×350.)

Fig. 4.—Forty-eight hour cortisone inflammation site with an incomplete and sparse leukocytic zone (*a*). No fibroblasts are seen at the periphery (*d*) and there is extension of the process into the adjacent muscle (*d'*). (H&E ×350.)

TABLE I. INDICATING TREATMENT AND TIME OF SACRIFICE OF THE VARIOUS GROUPS OF MICE THAT HAD BEEN INJECTED SUBCUTANEOUSLY WITH 0.025 ML. OF TURPENTINE

GROUP NO.	CORTISONE DOSAGE	TOTAL NO. MICE	NO. OF MICE SACRIFICED AT INDICATED INTERVALS POST-TURPENTINE INJECTION									
			3 HR.	6 HR.	12 HR.	1 DAY	2 DAYS	3 DAYS	4 DAYS	5 DAYS	7 DAYS	14 DAYS
1	none	73	7	7	7	7	7	7	7	7	7	10
2	1 mg. daily for three days prior to turpentine and daily until sacrifice	55	5	5	5	5	5	5	5	5	5	10
3	1 mg. daily three days after turpentine until sacrifice	40	0	0	0	0	0	0	10	10	10	10

Group 2 there were poorly defined areas of inflammation with some liquefaction necrosis of the cells of the exudate. The areas of inflammation were incompletely walled off from the adjacent tissue. Numerous and diffuse foci of scant inflammatory cells extended deeply into the adjacent muscle with beginning necrosis of muscle fibers.

On the fourteenth day Group 1 showed considerable abscess cavity contraction, few viable leukocytes, and a dense zone of granulation tissue while the adjacent muscle tissue was only occasionally and insignificantly involved. In Group 2, although considerable resolution was present, there were relatively more viable leukocytes and no definite granulation tissue capsules. Areas of muscle necrosis were present even in the absence of significant leukocytic infiltration. In some respects these latter sections resembled the picture of inflammation seen in patients with agranulocytosis (see Figs. 5 and 6).

In Group 3 the administration of cortisone modified the reaction so that it tended to approach that seen in Group 2. For example, the leukocytes present on the third day just prior to cortisone injection became necrotic, and very few new leukocytes appeared. The fourteen-day sections showed a picture somewhere in between the status of Group 1 and Group 2. The muscle layer contained necrosis to a lesser extent than in Group 2.

The pH of the exudate at twenty-four hours, three days, and six days was alkaline.

DISCUSSION

The suppression of all elements of the inflammatory response are in general agreement with the reported studies of Menkin,[14] Michael and Whorton,[13] and Dougherty and Schneebell.[10] The findings as to the degree of granulation formation around the inflammatory site are in agreement with those of Taubenhaus and Amronin.[15]

In one important aspect the findings in this study differ from those of Michael and Whorton.[13] This relates in particular to the question of the degree of tissue necrosis. These observers reported that the ultimate development of tissue necrosis was diminished in the cortisone-treated animal. The exact duration of time at which this observation was made was not clearly stated

In this study it was observed that although there was liquefaction necrosis of the polymorphonuclear leukocytes, tissue necrosis was greater in the cortisone-treated group. The failure to localize the lesion resulted in the formation of necrosis in the adjacent muscle. This was present as late as the fourteenth day (termination of experiment). It is this finding in particular which may be an important factor in the occasional occurrence of unexplained intestinal perforation that has been reported to occur while the patient is under cortisone therapy. It may also account for the apparent reactivation of peptic ulcers.

Moon and Tershakobec,[16] in preliminary studies on rabbits, failed to observe any cortisone suppression of the degree of inflammatory hyperemia edema, and leukocytic infiltration. However, Menkin[17] states that the explanation for this might be that cortisone will not suppress if the inflammatory exudate is at an

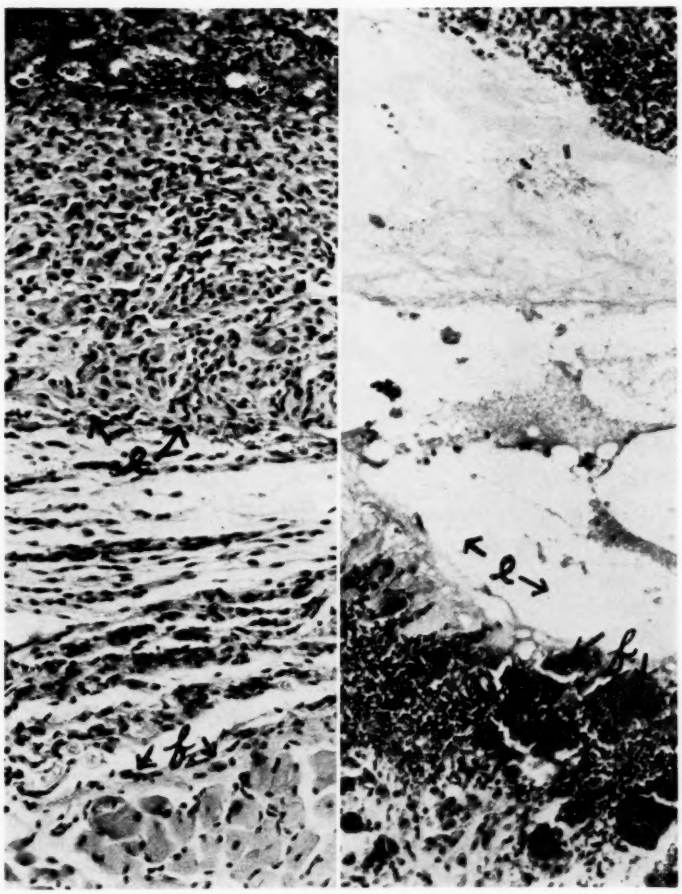


Fig. 5. Fig. 6.

Fig. 5.—Fourteen-day control inflammation site with intact muscle below (*f*) and well-formed granulation tissue zone above (*e*). (H&E ×350.)

Fig. 6.—Fourteen-day cortisone-treated inflammation site with necrosis of muscle and leukocytic infiltration below (*f*). There is some edema fluid and fibrin but no granulation tissue (*e*). (H&E ×350.)

acid level but that ACTH will. According to him, cortisone will suppress if the exudate is alkaline. The post-turpentine inflammatory exudate in this study was alkaline at least through the sixth day. This would be in keeping with Menkin's[17] observation that in an alkaline exudate cortisone prevents the leukotaxine from increasing capillary permeability. In a previous study,[18] suppression of turpentine-induced inflammation by cortisone was not observed. Further investigation indicates that in this previous study the turpentine dose was so great that the inflammatory response elicited was large enough to mask any cortisone suppressing effect.

The mechanism for adrenal corticosteroid suppression of inflammation is not entirely settled. Menkin[17] believes that interference with capillary permeability by inhibition of leukotoxin and exudin is of primary importance. This action may be mediated systemically or locally.[19] A cortisone-induced drop in the level of circulating fibrinogen may be a factor in the scant amount of fibrin seen at the inflammation site.[20] The effectiveness of the local inflammatory process is further interfered with by the decrease in the phagocytosing power of the polymorphonuclear leukocytes under the influence of cortisone.[21]

The combination of all these effects is probably not only responsible for many of the adverse events but the necrosis found in the later stages of acute inflammation may be the explanation for the so-called "rebound phenomenon" seen in postcortisone-treated patients with rheumatoid arthritis.

SUMMARY

Cortisone delays and suppresses all morphologic elements of chemically induced acute inflammation in mice.

Diffuse tissue necrosis at the inflammation site is present in the fourteenth day of inflammation in the cortisone-treated animals. This was not seen in the control animals.

The suppression of inflammation, the defective granulation tissue response, and in particular the presence of necrosis late in the acute inflammatory period are probably important mechanisms for many of the adverse effects seen with cortisone therapy.

REFERENCES

1. Symth, A. G.: Activation of Peptic Ulcer During Pituitary Adrenocorticotropic Hormone Therapy, J. A. M. A. **145**: 474-477, 1951.
2. Habif, D. V., Hare, C. C., and Glaser, G. H.: Perforated Duodenal Ulcers Associated with Pituitary Adrenocorticotropic Hormone (ACTH) Therapy, J. A. M. A. **144**: 996-997, 1950.
3. Personal observation.
4. Barrow, J. G., and Sikes, C. R.: Decubitus Ulcer in Rheumatic Fever Treated with Cortisone, J. A. M. A. **147**: 41-42, 1951.
5. Beck, J. C., Browne, J. S. L., Johnson, L. G., Kennedy, B. J., and MacKenzie, D. W.: Occurrence of Peritonitis During ACTH Administration, Canadian M. A. J. **62**: 423-425, 1950.
6. Rogan, C., Grokoest, A. W., and Boots, R. H.: Effect of Adrenocorticotropic Hormone (ACTH) on Rheumatoid Arthritis, Am. J. Med. **7**: 741-749, 1949.
7. King, E. Q., Johnson, J. B., Batten, G. S., and Henry, W. L.: Tuberculosis Following Cortisone Therapy, J. A. M. A. **147**: 238-240, 1951.
8. Popp, C. G., Ottosen, P., and Brasker, C. A.: Cortisone and Pulmonary Tuberculosis, J. A. M. A. **147**: 241-242, 1951.

9. Fred, L., Levin, M. H., Rivo, J. B., and Barrett, T. F.: Development of Active Pulmonary Tuberculosis During ACTH and Cortisone Therapy, J. A. M. A. **147:** 242-245, 1951.
10. Dougherty, T. F., and Schneebell, G. L.: Role of Cortisone in Regulation of Inflammation, Proc. Soc. Exper. Biol. & Med. **75:** 854-859, 1950.
11. Mogabgab, W. J., and Thomas, L.: Read before the Central Society for Clinical Research, 23rd annual meeting, Chicago, Nov. 3-4, 1950.
12. Schwartzman, G.: Enhancing Effect of Cortisone upon Poliomyelitis Infection (strain MEFI) in Hamsters and Mice, Proc. Soc. Exper. Biol. & Med. **75:** 835, 1950.
13. Michael, M., and Whorton, C. M.: Delay of the Early Inflammatory Response by Cortisone, Proc. Soc. Exper. Biol. & Med. **76:** 754-757, 1951.
14. Menkin, V.: Further Studies on the Effect of Adrenal Cortex Extract and Various Steroids on Capillary Permeability, Proc. Soc. Exper. Biol. & Med. **51:** 39, 1942.
15. Taubenhaus, M., and Amronin, G. D.: The Effects of the Hypophysis, Thyroid, Sex Steroids, and the Adrenal Cortex upon Granulation Tissue, J. LAB. & CLIN. MED. **36:** 7-18, 1950.
16. Moon, V. H., and Tershakobec, G. A.: The Trigger Mechanisms of Acute Inflammation, Am. J. Path. **27:** 693-695, 1951.
17. Menkin, V.: Further Studies on Mechanisms of Increased Capillary Permeability in Inflammation with the Aid of Cortisone and ACTH, Proc. Soc. Exper. Biol. & Med. **77:** 592-597, 1951.
18. Spain, D. M., and Molomut, N.: Biological Studies on Cortisone in Mice, Science **112:** 335-337, 1950.
19. Shapiro, R., Taylor, B., and Taubenhaus, M.: Local Effects of Cortisone on Granulation Tissue and the Role of Denervation and Ischemia, Proc. Soc. Exper. Biol. & Med. **76:** 854-857, 1951.
20. Spain, D. M., and Molomut, N.: Studies in the Mechanism of Cortisone Inhibition of Granulation Tissue Formation, Am. J. Path. **27:** 755, 1951.
21. Crepea, S. B., Magnin, G. E., and Seastone, C. V.: Effect of ACTH and Cortisone on Phagocytosis, Proc. Soc. Exper. Biol. & Med. **77:** 704-706, 1951.

PLASMA AND RED CELL CATALASE IN RHEUMATOID ARTHRITIS

M. R. JEFFREY, M.B., M.R.C.P.
BATH, ENGLAND

HYPOCHROMIC anemia is frequent in patients afflicted with rheumatoid arthritis. Inadequate hemoglobin production appears to be a most important factor in anemia of this type, and is associated with disordered iron metabolism (Nilsson, 1948; personal observations). It seemed of interest to investigate whether the activity and, therefore, possibly the production of the enzyme catalase, structurally a close relative of hemoglobin, was altered in the plasma or red cells of cases of rheumatoid arthritis.

MATERIAL AND METHODS

Fifty cases (20 male and 30 female) of active rheumatoid arthritis, free from any other disease liable to affect hemopoiesis, were investigated. Blood was taken between 9 and 10 A.M., the patients having remained at rest in bed after an overnight fast.

The activity of each patient's disease was classified clinically as slightly, moderately, or very active, according to the recent progress of the disease, the degree of weight loss and asthenia, and the evidence of active joint inflammation.

The following methods were used: catalase activity—Dille and Watkins (1949); hemoglobin—alkaline hematin, comparing photometrically with Gibson-Harrison artificial standard; serum iron—Kitzes, Elvehjem, and Schuette (1944).

RESULTS

1. *Plasma Catalase.*—

(a) *Controls:* Seven subjects (three normal, one with osteoarthritis, one with prolapsed disc, and two with fibrositis with iron deficiency anemia) gave results in the range 4.9 to 8.6, with a mean of 7.1 units. Dille and Watkins found a normal range of 4.2 to 9.2, with a mean of 6.9 units.

(b) *Rheumatoid arthritis:* The following values were obtained:

Men	Range 3.3 to 13.1.	Mean 6.5 units. S.D. 2.7
Women	Range 3.3 to 14.7.	Mean 7.0 units. S.D. 2.7
	All cases	Mean 6.8

The values were outside the normal range of Dille and Watkins in fifteen of the fifty cases, eight being low and seven high. The cases with abnormal values showed no particular features as regards age, sex, duration or activity of disease, or other clinical manifestations. The mean hemoglobin value of the "low catalase" group was 11.7, and of the "high" group was 13.0, the mean of the whole series being 11.6 Gm. per 100 milliliters.

Scatter diagrams (omitted to save space) show no correlation between plasma catalase and either age, duration of disease, hemoglobin level, or serum iron value. In the figures of Table I, no correlation is evident between plasma catalase and activity of the rheumatoid disease.

From the South-west and Oxford Regional Rheumatism Research Unit, Royal National Hospital for Rheumatic Diseases.
Received for publication, Oct. 15, 1951.

TABLE I

ACTIVITY GROUP		1.		2.		3.	
		M.	F.	M.	F.	M.	F.
Mean catalase activity	Plasma	4.8	7.0	6.9	7.0	6.6	8.4
	Cells	99.1	96.2	98.0	97.6	99.5	100
No. of cases		4	10	15	19	1	1
Mean catalase activity—both sexes	Plasma	6.4		6.95		7.5	
	Cells	97.0		97.8		99.8	

2. *Red Cell Catalase.*—

(a) *Controls:* Dille and Watkins did not quote their figures for normal values; using a 1:500 dilution of packed red cells and following their technique exactly, our controls gave values ranging between 85 and 104 units with a mean of 95.2.

(b) *Rheumatoid Arthritis:* The following values were obtained:

Men	Range 88 to 104.	Mean 98.3 units.	S.D. 4.3
Women	Range 85.5 to 108.	Mean 97.2 units.	S.D. 5.1
	All cases	Mean 97.7 units.	

The figures from the rheumatoid cases were thus very similar, both in range and in their mean value, to those of the controls. No correlation existed between the catalase activity and the hemoglobin content of the red cells. Catalase activity was in fact uninfluenced by the frequently lowered hemoglobin content of the cells. Activity of the rheumatoid disease did not appear to influence cell catalase activity (Table I); age, sex, duration of disease, and serum iron value were also unrelated.

COMMENT

The method of Dille and Watkins, employed here, estimates catalase activity in terms of iodine liberated from potassium iodide by oxygen resulting from the enzymatic breakdown of hydrogen peroxide in buffered solution; the results are expressed in arbitrary units. These authors have given evidence that the method does in fact estimate the activity of catalase and not of other enzymes.

The catalase content of red cells is vastly greater than that of plasma and it is therefore difficult to prove that the observed plasma catalase activity is not in fact due to liberation of red cell catalase by slight damage to cells during the drawing of blood. Certainly the degree of hemolysis caused by drawing air bubbles into the syringe while taking blood or by any "squirting" during transfer of blood from syringe to tube is enough to raise the plasma catalase outside the normal range. Nevertheless, normal values lie within a small range (Dille and Watkins, loc. cit.; Perlmann and Lipmann, 1946) and individuals tend to exhibit fairly constant day-to-day values. It seems that,

in normal people, plasma or serum catalase activity may well result from liberation of the enzyme during the normal processes of destruction of aged red cells.

(a) *Plasma Catalase.*—In the cases of rheumatoid arthritis studied here, the mean value was almost identical with that of Dille and Watkins' normal subjects, although the range was rather greater in the arthritic cases. This observation, coupled with the lack of correlation between plasma catalase values and either disease activity, hemoglobin level, or other factors, indicates that the plasma catalase is essentially unaltered in rheumatoid arthritis.

(b) *Red Cell Catalase.*—The range and mean values for red cell catalase in the rheumatoid cases were very close to those of the control subjects. No correlation was apparent between catalase values and either the duration or activity of disease, the hemoglobin content of the red cells, or the serum iron concentration. It is of interest that the catalase value was not reduced in the presence of marked reduction in the corpuscular hemoglobin. There is no evidence, therefore, of parallel disturbances in hemoglobin and catalase production.

SUMMARY

Plasma and red cell catalase has been estimated in fifty cases of active rheumatoid arthritis.

Essentially normal values were discovered both in anemic and nonanemic cases.

My thanks are due to Dr. G. D. Kersley, Director of the South-west and Oxford Regional Rheumatism Research Unit, Bath, for permission to investigate patients under his care.

REFERENCES

Dille, R. S., and Watkins, C. H.: A Method for Determination of Plasma Catalase and Values Obtained in Normal Adults, J. Lab. & Clin. Med. 33: 480-486, 1948.

Kitzes, G., Elvehjem, C. A., and Schuette, H. A.: Determination of Blood Plasma Iron, J. Biol. Chem. 155: 653-660, 1944.

Nilsson, F.: Anaemia Problems in Rheumatoid Arthritis, Acta Med. Scandinav., Supp. 120, 121 (accompanies Vol. 130), 1948.

Perlmann, G. E., and Lipmann, F.: Catalase in Serum and Plasma, Arch. Biochem. 7: 159, 1945.

ELECTROLYTE CHANGES PRODUCED BY ACTH, CORTISONE, AND DOCA IN CIRRHOSIS OF THE LIVER

WILLIAM H. BLAHD, M.D., WILLIAM S. ADAMS, M.D., ALAN LESLIE, M.D., RALPH GOLDMAN, M.D., AND SAMUEL H. BASSETT, M.D.
LOS ANGELES, CALIF.
WITH THE TECHNICAL ASSISTANCE OF TOMOKO FUKUI

THE mechanism of formation of ascites and edema in portal cirrhosis of the liver has been a subject of discussion for many years. In general, four main mechanisms have been advanced to explain the accumulation of fluid in this disease: (1) portal hypertension, (2) reduced osmotic pressure of the blood plasma, (3) increased secretion or decreased destruction of an antidiuretic hormone, and (4) sodium retention. None of these concepts alone affords a completely adequate explanation of the clinical picture. Although the authors believe that the first three hypotheses play important roles in the production of ascites, no attempt will be made to evaluate them in this paper. Rather, emphasis will be placed on the exchange of sodium in cirrhosis, the effect of certain steroids which appear to influence sodium metabolism, and the possible relationship of sodium exchange to adrenal cortical activity.

METHOD

Six patients, with advanced hepatic cirrhosis and ascites as evidenced by history, physical examination, liver function tests (see Table I), and liver biopsy, and three normal individuals were selected for detailed study on the metabolism ward. During the period of observation all subjects received an 800 mg. sodium diet.* Fluid intake was restricted to a total of 1,500 ml. daily. Twenty-four hour collections of urine were analyzed daily for sodium and potassium, using the Beckman flame photometer. The twenty-four hour neutral lipid-soluble reducing substances of the urine (11-oxysteroid excretion) of five of the patients were determined.[1] Frequent determinations of blood sodium, potassium, and plasma proteins were made during the course of study. These particular data are not included, however, since they showed no significanct fluctuation. Sweat tests[2] were performed on all subjects during the first five days of each week, and sweat samples were analyzed for sodium and potassium.

Each subject received the following substances by intramuscular injection in individual ten-day courses: desoxycorticosterone acetate (DOCA), 25 mg.; 17 hydroxy, 11 dehydrocorticosterone (cortisone), 200 mg.; cortisone, 200 mg., and DOCA, 25 mg.; and adrenocorticotrophic hormone (ACTH), 100 mg. Between courses of injections there were intervals of not less than ten days. Case 4 (Fig. 4, *A*) received a preliminary course of DOCA, 10 mg. daily, for a period of twenty-five days. The metabolic effects in this patient were inconclusive, consequently larger doses of DOCA were employed during this and subsequent investigations.

Reviewed by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions of the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

Received for publication, Oct. 15, 1951.

From the Metabolic Unit, General Medical Research Program, Veterans Administration Center, and the Department of Medicine, University of California.

*By actual analysis, the daily total sodium content of the diet varied from 800 to 1,000 milligrams.

TABLE I. CLINICAL AND LABORATORY DATA ON PATIENTS STUDIED *

	CASE	AGE	ASCITES	EDEMA	PLASMA PROTEINS			SODIUM (MEQ.)	POTASSIUM (MEQ.)	SERUM BILIRUBIN (MG./%)	BSP RETENTION	CEPH. FLOC.	THY. FLOC.	THY. TURB.	24-HOUR 11-OXY. EXCRETION (MG.)
					ALBUMIN (GM./%)	GLOBULIN (GM./%)	TOTAL (GM./%)								
1.	G. C.	49	3+	1+	3.4	3.1	6.5	130	3.9	.47	10.8%	4+	4+	20	- -
2.	O. L.	52	4+	4+	3.2	1.6	4.8	133	4.4	.52	9.2%	2+	N	1	2.33*
3.	O. R.	55	3+	1+	3.4	2.9	6.3	137	4.4	.11	8.0%	2+		7	2.59*
4.	E. L.	63	3+	1+	2.9	2.9	5.8	139	4.0	.9	9.2%	2+		26	1.99*
5.	A. R.	55	4+	4+	2.5	4.2	6.7	136	4.9	.3	5.0%	3+	3+	8	4.09*
6.	R. M.	50	4+	4+	2.7	4.8	7.5	133	4.0	.8		4+	4+	18	2.12

*Average of two determinations.

RESULTS

Four of the patients with cirrhosis and ascites exhibited a definite biochemical and clinical response to the administration of DOCA, cortisone, and ACTH. The two most advanced cases, however, showed little or no response to these substances.

Effects of DOCA and Cortisone.—Figs. 1 to 4 are charts of the data obtained from the study of the four patients who responded to DOCA and cortisone. It is readily seen that the administration of DOCA or cortisone resulted in a marked decrease in the concentration of sweat sodium and in the amount of sodium excreted in the urine. This effect persisted, in most instances, during the entire course of administration of the above substances, and was followed

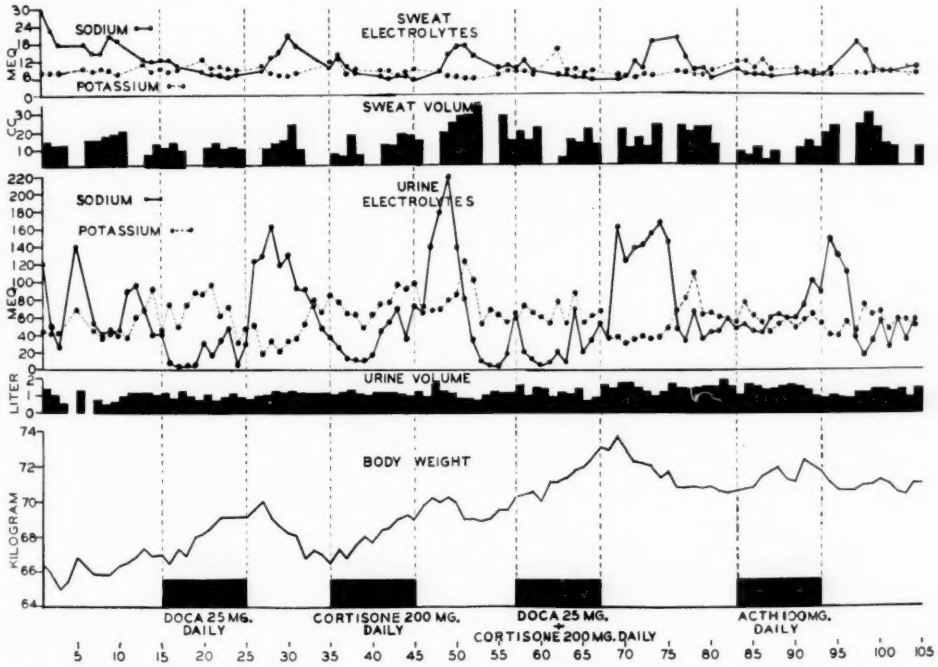


Fig. 1.—Case G. C. (Values for sweat and urine electrolytes are expressed as milliequivalents per liter.)

by a prompt and substantial withdrawal diuresis of sodium and water. Case G. C. (Fig. 1) differed in one respect from the others in that there appeared to be an escape from the salt-conserving influence of DOCA and cortisone. This was reflected by a return of the urinary sodium excretion to control levels, despite continuous administration of the test material. Nevertheless, the usual prompt withdrawal diuresis of sodium and water was observed.

Simultaneous Administration of DOCA and Cortisone.—The simultaneous administration of DOCA and cortisone produced a marked diminution in urine and sweat sodium. In three instances (Figs. 1, 3, and 4*B*) there appeared to be a gradual escape from this effect, manifested particularly by a gradual rise

in the urine sodium excretion to levels exceeding control values. Following withdrawal of DOCA and cortisone a prompt diuresis occurred which appeared to be somewhat more pronounced than the one subsequent to separate administration of these steroids.

Effects of ACTH.—In Cases 3 and 4 the administration of ACTH also produced a marked diminution of sweat sodium concentration and urine sodium excretion which was followed by a marked withdrawal diuresis of urine sodium and an increase in sweat sodium concentration (Figs. 3 and 4*B*). Cases 1 and 2 (Figs. 1 and 2) exhibited a gradual escape from this salt-retaining effect, with

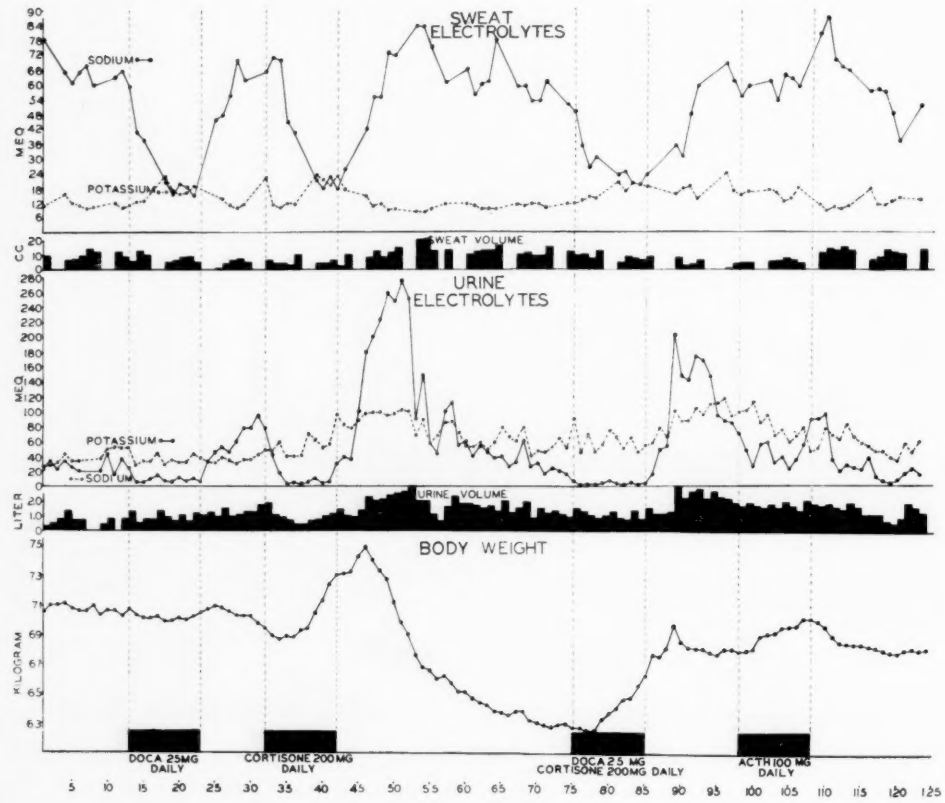


Fig. 2.—Case O. L.

a rise in excretion of urine sodium to higher than control levels. This was followed by a moderate withdrawal diuresis of sodium and a more concentrated sweat sodium. It should be noted that the sweat sodium concentration in Case 2 (Fig. 2) was not significantly affected by ACTH administration.

Sweat and Urine Potassium.—Sweat potassium remained remarkably constant during the administration of DOCA, cortisone, and ACTH in both cirrhosis patients and normals. Urine potassium, on the contrary, exhibited considerable and inconstant variation during the entire period of observation. A

trend was noted in two instances: the withdrawal of cortisone in three cases, 2, 3, and 4 (Figs. 2, 3, and 4*B*), was followed by a moderate potassium diuresis; the withdrawal of ACTH in two patients (Cases 3 and 4) and two controls (Cases 7 and 8) (Figs. 3, 4*B*, 7, and 8) was associated with the prompt retention of potassium.

Urine and Sweat Volumes.—The variations in urine and sweat volumes during the course of study corresponded to the changes observed in sweat and urine sodium. During the administration of DOCA, cortisone, and ACTH the volumes of urine and sweat decreased, and upon withdrawal of these medications the volumes were observed to increase. In some instances, the loss of water appeared to exceed the amount retained during the administration of the above substances.

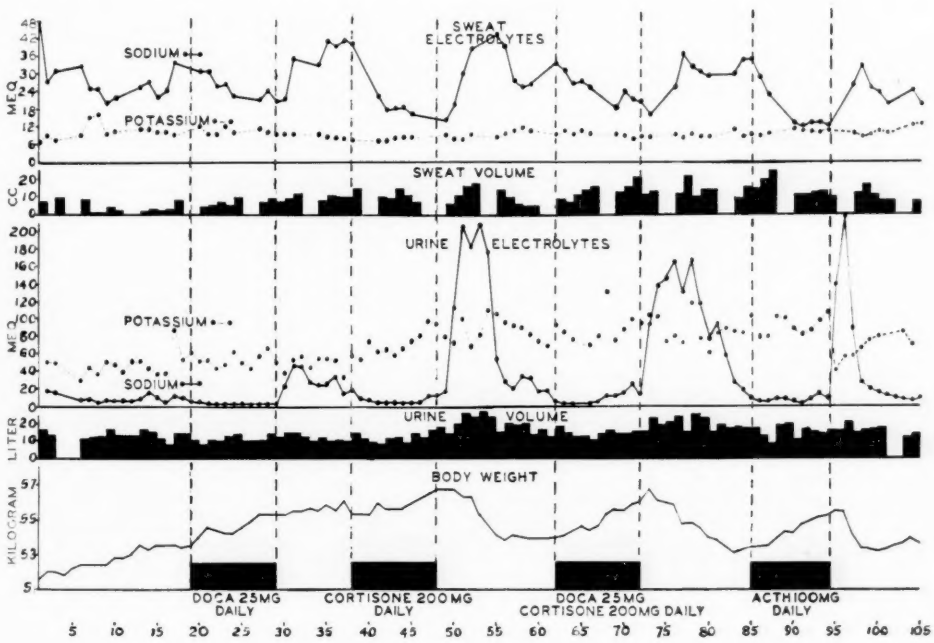


Fig. 3.—Case O. R.

Observations in Far-Advanced Cirrhosis.—Cases 5 and 6 (Figs. 5 and 6), the two patients with most advanced disease, differed in several respects from the four cases previously described. They maintained a base line urinary sodium excretion well below their daily intake. In general, there was little or no response to steroid or ACTH administration. Instead, a progressive and persistent gain in weight occurred which appeared to be accelerated by the administration of ACTH or adrenal cortical substances. The phenomenon of withdrawal diuresis was not observed. Both patients ultimately required paracenteses because of the persistent accumulation of ascitic fluid. Case 6 expired prior to the completion of the investigation following a severe hemorrhage from a ruptured esophageal varix. Necropsy revealed far-advanced portal cirrhosis.

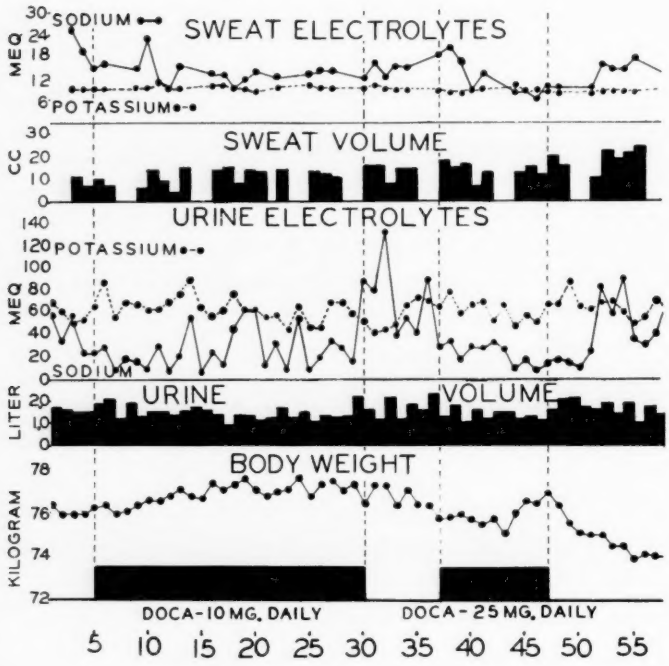


Fig. 4A.—Case E. L.

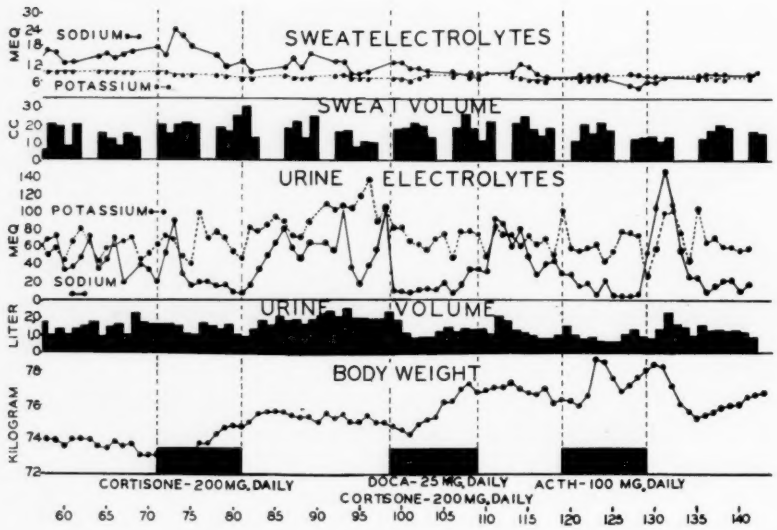


Fig. 4B.—Case E. L.

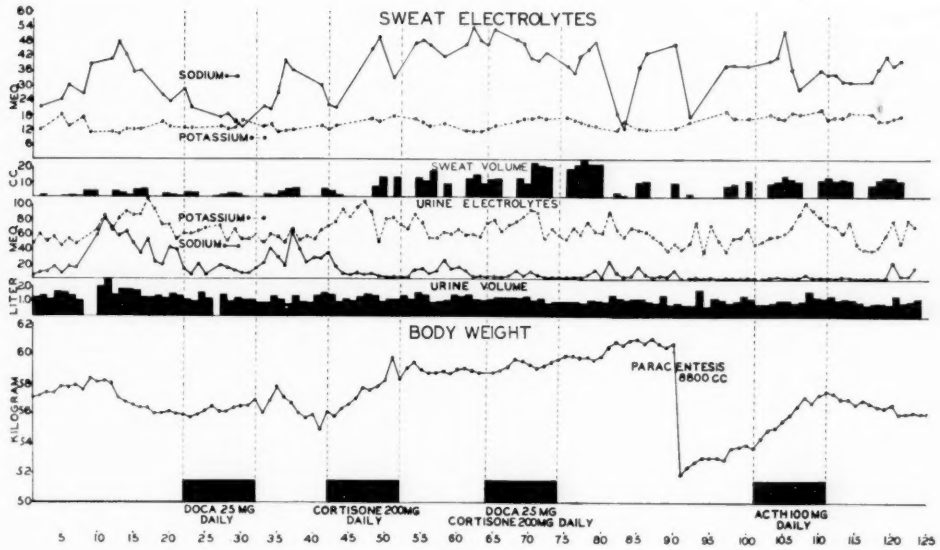


Fig. 5.—Case A. R.

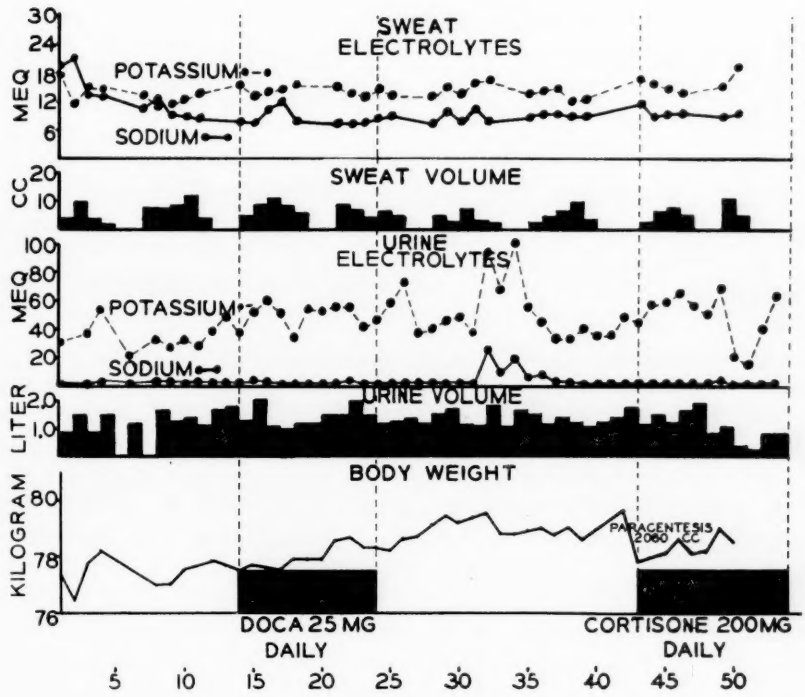


Fig. 6.—Case R. M.

Control Studies.—Control studies were performed on three individuals (Cases 7, 8, and 9) who were in good health except for stabilized orthopedic disabilities (Figs. 7 and 8). The electrolyte and body weight changes during steroid and ACTH administration were very similar to those observed in the first four patients, especially Cases 2, 3, and 4. (Figs. 2, 3, and 4.) Case 8 (control), in contrast to the preceding control studies, manifested a tendency to escape from the influence of cortisone, combined DOCA and cortisone, and ACTH, and to develop a gradual sodium diuresis, despite the continuous administration of test material (Fig. 8). His response, including the sweat electrolyte changes, resembled those seen in Case 1. (Fig. 1.) The alterations in body weight in this subject, however, were entirely dissimilar to any previously noted. The transient gain in weight, usually associated with the administration of steroids and ACTH, was not observed, instead there was a gradual and progressive loss of weight during the period of observation.

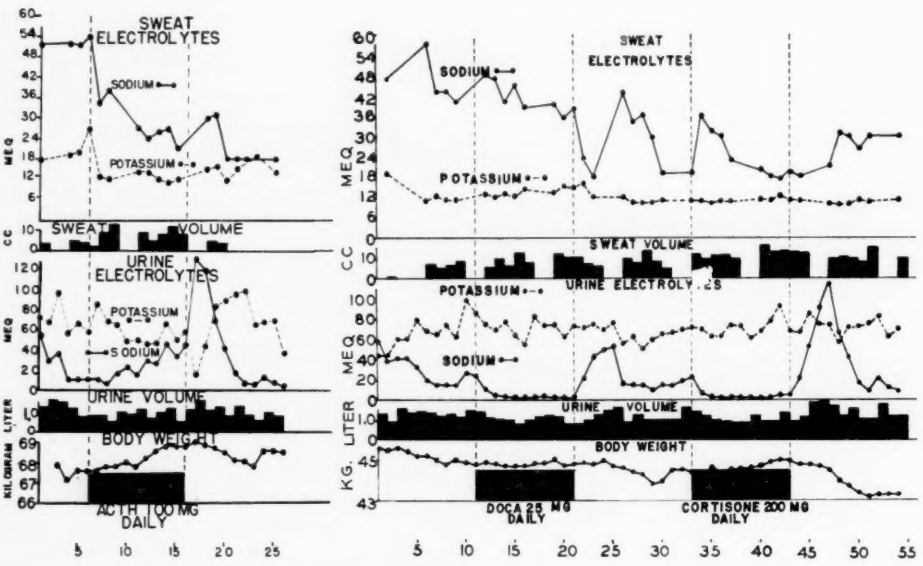


Fig. 7.—Cases 7 and 9.

DISCUSSION

Many investigators[3-7] have demonstrated conclusively that sodium retention occurs in cirrhosis with ascites. Moreover, it is well known that, regardless of the amount or route of administration, practically all sodium is diverted to the ascitic fluid and only minute amounts appear in the urine.[4, 6] This retention of sodium does not appear to stem from impaired renal function, for Farnsworth and Krakusin[5] and Patek and associates[2] have obtained normal renal clearance values in such patients. In the opinion of Goodyer and co-workers,[7] the retention is the result of increased tubular reabsorption of sodium which, according to Faloon and collaborators[6] and Leaf and Couter,[9] is probably regulated by the

adrenal cortex. As suggested by Faloon, augmented adrenal cortical activity may be responsible for the extreme sodium retention which occurs in advanced cirrhosis.

From the data obtained in this study, it would appear that most patients with cirrhosis and ascites respond in a relatively normal fashion to exogenous ACTH and certain adrenal cortical "salt-active" substances. Furthermore, both the initial concentration of sweat sodium, with a single exception, and the "11-oxysteroid excretion" was found to be within the normal range.

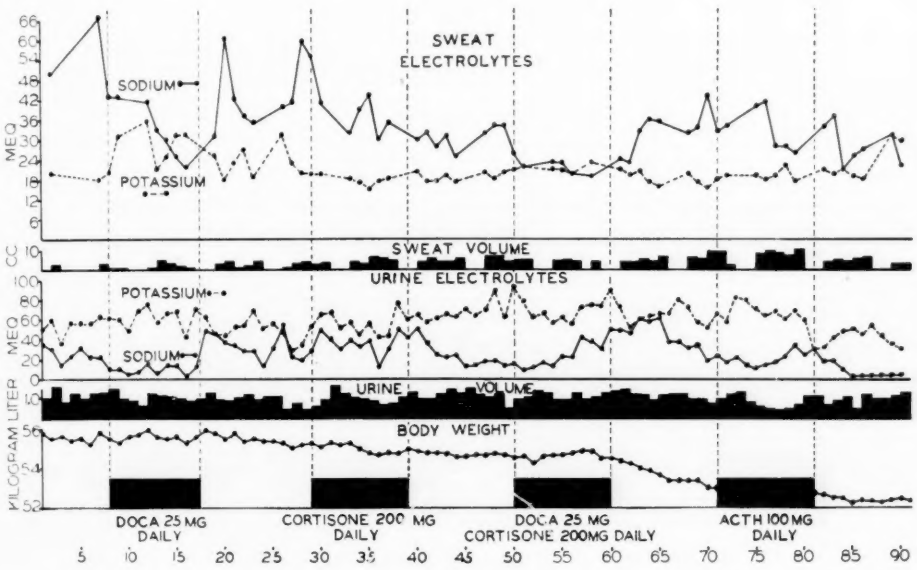


Fig. 8.—Case 8.

Although the weight of evidence would seem to support the thesis that adrenal cortical function is normal in the majority of patients with cirrhosis, the possibility of cortical hyperfunction cannot be excluded with certainty, and some correlation may exist between the severity of the disease and the secretion of adrenal "salt-active" substances. In this regard, it seems pertinent that in the most severely ill patients (Figs. 5 and 6), the excretion of sodium in the urine was persistently low, and there was little in the way of clinical or biochemical response to the administration of ACTH and adrenal steroids. This might be interpreted as indicative of a pre-existing maximum effect of "salt-active" steroids on the renal tubules. One of these men, Case 6 (Fig. 6), had an initial sweat sodium concentration well within the range reported for Cushing's syndrome,[10] and the other, Case 5 (Fig. 5), the highest urinary "11-oxysteroid" value obtained in the study (Table I). Moreover, the fact that four patients were capable of responding in an essentially normal manner to exogenous steroids and ACTH does not necessarily exclude adrenal cortical hyperactivity. Lewis and Wilkins[11] have shown that individuals with Cushing's syndrome are capable of responding to ACTH in much the same manner as normals, with an increased

output of urinary steroids and retention of sodium. The question of whether adrenal cortical function is normal or increased in cirrhosis cannot be decided on the basis of the data at hand.

On the other hand, the possibility of an antagonistic renal effect of cortisone and DOCA has been suggested by Forsham and associates.[12] They have confirmed the work of Soffer,[13] and demonstrated that some individuals with Cushing's syndrome are relatively refractory to the salt-retaining action of DOCA, whereas patients with Addison's disease, on maintenance doses of DOCA, undergo a sodium diuresis when cortisone is administered. These authors[12] suggest ". . . that the DOCA-fastness of certain patients with Cushing's syndrome may be due to the blocking action on the renal tubule of an excess of adrenal steroids of the 11-, 17-oxysteroid type." The effect of the simultaneous administration of DOCA and cortisone, observed in this study, was an initial sodium retention with a subsequent limited release of urinary sodium. This was followed by a prolonged and extensive sodium diuresis, upon abrupt withdrawal of the steroids (Figs. 1, 3, 4*B*, and 8). The interaction of DOCA and cortisone is certainly not clear, since initially their combined effect is comparable to the effect of either substance administered individually. Yet, in some instances, the ensuing withdrawal diuresis is more prolonged than would be expected following the individual administration of either steroid. Furthermore, the limited release of urinary sodium, which occurs during the simultaneous administration of these substances, does not occur, ordinarily, when either one of the steroids is used individually. The results obtained in this study, therefore, suggest that a "competitive renal action" may exist between DOCA and cortisone, and that this phenomenon may be observed in cirrhosis with ascites as well as in normal individuals.

The effect of DOCA, cortisone, and ACTH on the majority of the patients with cirrhosis was favorable. This was especially true of the latter two hormones which produced a marked increase in appetite and well-being resulting in a significant improvement in nutrition and consequent real weight gain. Four patients exhibited a marked sodium diuresis following the cessation of DOCA, cortisone, and ACTH. The withdrawal diuresis was sufficiently pronounced in several cases to bring about the complete disappearance of all edema and ascites. Thorn[14] and Eliel[15] suggest that this withdrawal phenomenon may be the result of a temporary depression of the adrenal or pituitary gland resulting in a state of relative adrenal cortical insufficiency. The possibility of employing such salt-active substances as a means of producing diuresis in cirrhosis of the liver with ascites deserves further consideration.

SUMMARY AND CONCLUSIONS

1. The effect of ACTH and certain steroids, which appear to influence the exchange of sodium and water, were studied in six patients with cirrhosis of the liver and three normal subjects. The relationship of adrenal cortical activity to the exchange of sodium was also investigated.

2. Four patients with cirrhosis responded in a normal fashion to ACTH and adrenal cortical substances. The concentrations of sweat sodium and urinary "11-oxysteroid excretion" were within normal range in these cases.

3. In patients with far-advanced disease, the administration of DOCA, cortisone, and ACTH accelerated the development of ascites and edema, whereas patients with less advanced disease manifested only transitory salt and water retention.

4. Results obtained from the simultaneous administration of DOCA and cortisone suggested the existence of a competitive renal action between these substances.

5. A marked withdrawal diuresis of sodium and water followed the discontinuation of DOCA, cortisone, and ACTH which was sufficiently pronounced in several cases to resolve all edema and ascites.

REFERENCES

1. Heard, R. H. D., Sobel, H., and Venning, E. H.: The Neutral Lipid-Soluble Reducing Substances of Urine as an Index of Adrenal Cortical Function, J. Biol. Chem. **165**: 699, 1946.
2. Leslie, A., and Levin, M. H.: Sweat Test Simplified for Clinical Use, Am. J. Med. **8**: 823, 1950.
3. Combined Staff Clinic: Mechanisms of Ascites Formation, Am. J. Med. **9**: 102, 1950.
4. Gabuzda, G. J., Jr., Treager, H. S., and Davidson, C. S.: Hepatic Cirrhosis. Factors Contributing to the Failure to Excrete Urinary Sodium During the Accumulation of Ascites and Edema, J. Clin. Investigation **29**: 814, 1950.
5. Farnsworth, E. B., and Krakusin, J. S.: Electrolyte Partition in Patients with Edema of Various Origins, J. Lab. & Clin. Med. **33**: 1545, 1948.
6. Faloon, W. W., Eckhardt, R. D., Cooper, A. M., and Davidson, C. S.: The Effect of Human Serum Albumin, Mercurial Diuretics, and a Low Sodium Diet on Sodium Excretion in Patients with Cirrhosis of the Liver, J. Clin. Investigation **28**: 595, 1949.
7. Goodyer, A. V. N., Relman, A. S., Lawrason, F. D., and Epstein, F. H.: Salt Retention in Cirrhosis of the Liver, J. Clin. Investigation **29**: 973, 1950.
8. Patek, A. J., Jr., Mankin, H., Colcher, H., Lowell, A., and Earle, D. P., Jr.: The Effects of Intravenous Injection of Concentrated Human Serum Albumin upon Blood Plasma, Ascites, and Renal Functions in Three Patients with Cirrhosis of the Liver, J. Clin. Investigation **26**: 1178, 1947.
9. Leaf, A., and Couter, W. T.: Evidence That Renal Sodium Excretion by Normal Human Subjects Is Regulated by Adrenal Cortical Activity, J. Clin. Investigation **28**: 1067, 1949.
10. Conn, J. W.: Electrolyte Composition of Sweat, Arch. Int. Med. **83**: 416, 1949.
11. Lewis, R. A., and Wilkins, L.: The Effect of Adrenocorticotrophic Hormone in Congenital Adrenal Hyperplasia with Virilism and in Cushing's Syndrome Treated with Methyl Testosterone, J. Clin. Investigation **28**: 394, 1949.
12. Forsham, P. H., Flink, E., Emerson, K., Jr., and Thorn, G. W.: Metabolic Studies on Cushing's Syndrome, J. Clin. Investigation **28**: 781, 1949.
13. Soffer, L. J., Lesnick, G., Sorkin, S. Z., Sobotka, H. H., and Jacobs, M.: The Utilization of Intravenously Injected Salt in Normals and in Patients with Cushing's Syndrome Before and After Administration of Desoxycorticosterone Acetate, J. Clin. Investigation **23**: 51, 1944.
14. Thorn, G. W., Merrill, J. P., Smith, S., III, Roche, M., and Frawley, T. F.: Clinical Studies with ACTH and Cortisone in Renal Disease, Arch. Int. Med. **80**: 319, 1950.
15. Eliel, L. P., Pearson, O. H., and West, C. D.: Excessive Losses of Sodium, Chloride, and Water After Administration of Cortisone or ACTH, Suggesting Transient Adrenocortical Insufficiency, J. Clin. Investigation **30**: 637, 1951.

THE ESTIMATION OF INSULIN SENSITIVITY* BY THE MODIFIED GLUCOSE INSULIN TOLERANCE TEST

SYDNEY S. LAZARUS, M.D., AND BRUNO W. VOLK, M.D.
BROOKLYN, N. Y.

HIMSWORTH[1] in 1936 suggested that an insulin-insensitive type of diabetes mellitus might exist due not to lack of insulin but rather to "lack of an unknown factor or condition which renders the body sensitive both to injected or pancreatic insulin." The Insulin Tolerance Test (I.T.T.) was found to be unsuitable for the detection of this type of diabetes, since the results obtained by this method are not comparable except when they are from the same individual, and even then only if the starting blood sugars are approximately the same.

The inadequacy of the I.T.T. as a test for insulin sensitivity has also been borne out by the recent work of Somogyi,[2] who observed that when the blood sugar is reduced below the fasting level, counter-regulatory mechanisms to the induced hypoglycemia are set in motion which influence the rate and extent of the fall of the blood sugar and the time of the return to the fasting value. To obviate these difficulties, Himsworth introduced the Glucose Insulin Tolerance Test (G.I.T.T.) which consists of the simultaneous administration of oral glucose and intravenous insulin. He reported that this test results in a flat blood sugar curve in the normal individual and in a sizeable percentage of diabetic patients ("insulin-sensitive type"), whereas in other diabetic patients ("insulin-insensitive type"), a curve resembling the routine glucose tolerance curve is noted, and he was therefore able to divide diabetic patients into two groups.

An an amplification of the original procedure, Himsworth and Kerr[3] utilized the ratio of the area included by the glucose insulin tolerance curve to the area included by the glucose tolerance curve, the I/G ratio, as an index of insulin sensitivity, and thus gave further support to the division of diabetic patients into the two groups: insulin sensitive and insulin insensitive.

This was in contrast to the conclusions of DeWesselow and Griffiths,[4] who used a modification of Himsworth's original technique and could find no sharp division between insulin-sensitive and insulin-insensitive types of diabetic patients, but rather a general distribution of responses similar to that in a normal group of subjects.

Subsequently, the G.I.T.T. was utilized by Fraser, Albright and Smith[5] to differentiate whether the hyperglycemia unresponsiveness elicited by the Glucose Tolerance Test (G.T.T.) in diabetic patients is due to insulin lack or insulin resistance. They concluded that in hypoinsulin diabetes the G.I.T.T. will be normal, whereas in diabetes due to hyperadrenocorticism and hyperpituitarism, a curve similar to the G.T.T. will be found. These authors, however, did not consider the role of the liver as a pathogenetic factor in the production of insulin-resistant diabetes, as has been suggested by other investigators.[6-8]

Recently Kirketerp[9] utilized the I/G ratio to restudy the problem of insulin sensitivity in diabetic patients. He was unable, however, to divide his patients into two distinct groups, since the distribution of the values obtained made it probable that the patients comprised a single group.

---

*The term insulin sensitivity is used in the sense of the effect of insulin on the rate of withdrawal of glucose by the tissues from the extracellular fluid compartment, having no reference to the depth of decline of the blood sugar level.

From the Division of Laboratories and the Department of Medicine, Jewish Sanitarium and Hospital for Chronic Diseases, Brooklyn, New York.

Received for publication, Sept. 13, 1951.

In a study of the G.I.T.T. in nondiabetic individuals, we observed that six of seventeen had an abnormal pattern, even though there was no other clinical or laboratory evidence of an endocrine or metabolic abnormality. The apparent lack of specificity of this procedure thus revealed is supported by a previous finding[5] that in patients with acromegaly and diabetes, the G.I.T.T. may be of the type of either insulin sensitivity or insulin resistance, even though theoretically only the latter response should be obtained.

Because of the variability of the results of the Glucose Insulin Tolerance Test in the normal individual, and because of the discrepancies of the findings regarding the differentiation of the two types of diabetic patients, we have attempted to substitute a modification of the G.I.T.T. in which a standard dose of glucose is administered intravenously followed thirty minutes later by intravenous insulin instead of the simultaneous administration of oral glucose and intravenous insulin. The present report embodies experimental data showing that this method gives a uniform type of response in the normal individual. It was hoped that it would therefore be a better method than the original procedure for the differentiation of insulin resistance from insulin sensitivity.

MATERIAL AND METHODS

The experimental subjects consisted of two groups: (a) forty fasting nondiabetic volunteers consisting of hospital employees and laboratory workers, and (2) twenty-four fasting hospitalized diabetic patients who had not received any insulin for forty-eight hours prior to the test procedures and whose associated clinical conditions are registered in Table I. Each of these two groups was subjected to the standard intravenous G.T.T. and the modified G.I.T.T. For the G.T.T. 25 Gm. of glucose in 50 per cent solution were injected intravenously. Immediately before and at intervals of thirty, sixty, one hundred and twenty, and one hundred and eighty minutes after the injection, capillary blood samples were collected for the glucose determinations.

In carrying out the modified G.I.T.T., 25 Gm. of glucose in 50 per cent solution were given intravenously followed after 30 minutes by 0.1 unit of crystalline insulin per kilogram of body weight intravenously. Capillary blood samples for glucose determinations were withdrawn at fasting, and at thirty, fifty, sixty, seventy-five, ninety, one hundred and twenty, one hundred and fifty, and one hundred and eighty minutes after the administration of glucose. The blood sugar determinations were done by the Nelson modification of the Folin-Wu Micro Method.[10]

RESULTS

The criteria for a normal intravenous G.T.T. were a fasting capillary blood sugar value not higher than 140 mg. per cent with a return of the one-hundred twenty-minute specimen to or below the fasting value.[11] These norms were met by all the controls, but by none of the diabetic patients.

Since in the modified G.I.T.T. all the 40 nondiabetic individuals showed a decline of the blood sugar level to fasting value within seventy-five minutes after the injection of glucose (forty-five minutes after insulin), this timing is taken as the criterion for normalcy for this procedure. Fig. 1, *A* demonstrates the mean curve in these individuals as compared to the mean of their Glucose Tolerance curve. Of the twenty-four diabetic patients, seven reached the fasting level within ninety minutes, four within one hundred twenty minutes, two within one hundred fifty minutes, and one within one hundred eighty minutes.

Figure 1, *B* is an example of the type of G.I.T.T. observed in this group of individuals as compared to the G.T.T. All of the remaining ten patients showed a decline of the blood sugar to the fasting level within seventy-five minutes. Fig. 1, *C* illustrates the curve obtained in one of these patients as compared with that of the G.T.T.

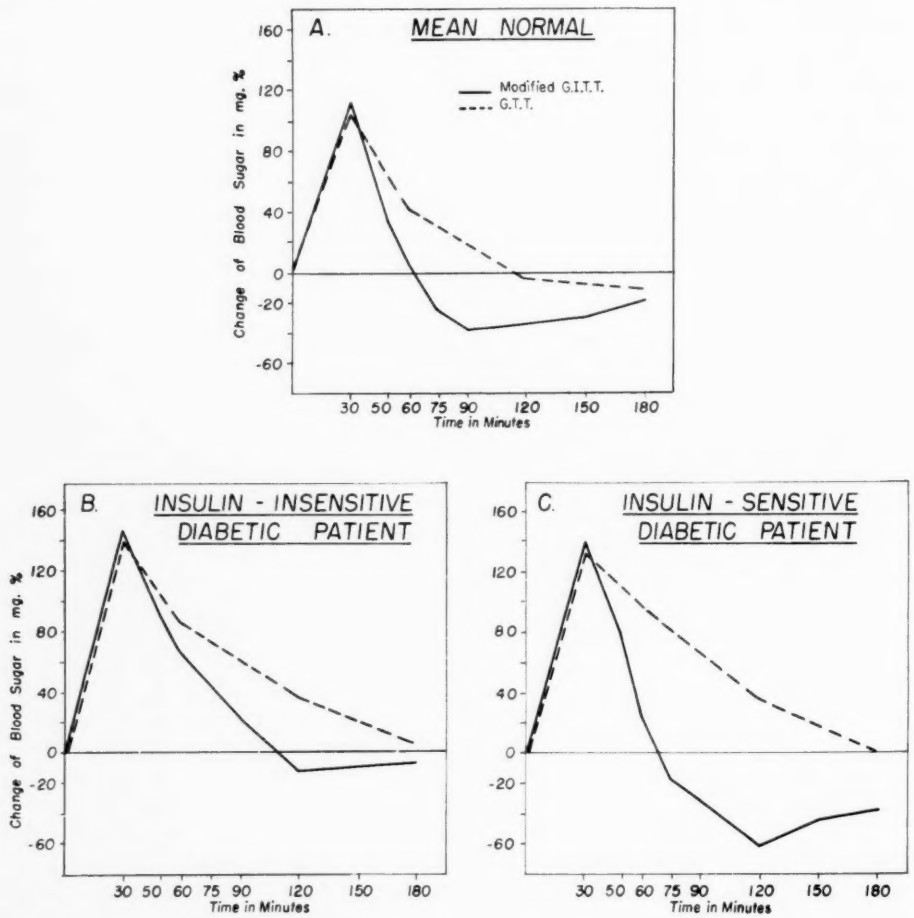


Fig. 1.—A comparison of the mean normal response (forty individuals) to the G.T.T. and modified G.I.T.T. with the responses of an insulin-sensitive and an insulin-insensitive diabetic patient.

In an attempt to develop a more exact mathematical statement for insulin sensitivity or resistance, the rate of decline of the blood sugar level was calculated for the thirty-minute period after the injection of insulin. In the forty nondiabetic individuals, the mean rate of decline of the blood sugar for this period was found to be 3.8 mg. per 100 ml. per minute, with a standard deviation of 0.7 mg. per 100 ml. per minute. Fig. 2 demonstrates the distribution of the rates of decline of the blood sugar level of the control individuals as compared to the diabetic patients. The slowest rate of decline in the nondiabetic persons

was 2.4 mg. per 100 ml. per minute, and the fastest 5.7 mg. per 100 ml. per minute. The rate of decline in seventeen of the diabetic patients (71 per cent) fell within the normal range. The remaining 7 (29 per cent) had a rate of decline of the blood sugar varying from 1.3 to 1.9 mg. per 100 ml. per minute. A comparison of the height of the fasting or thirty-minute blood sugar level with the rate of decline did not show any correlation.

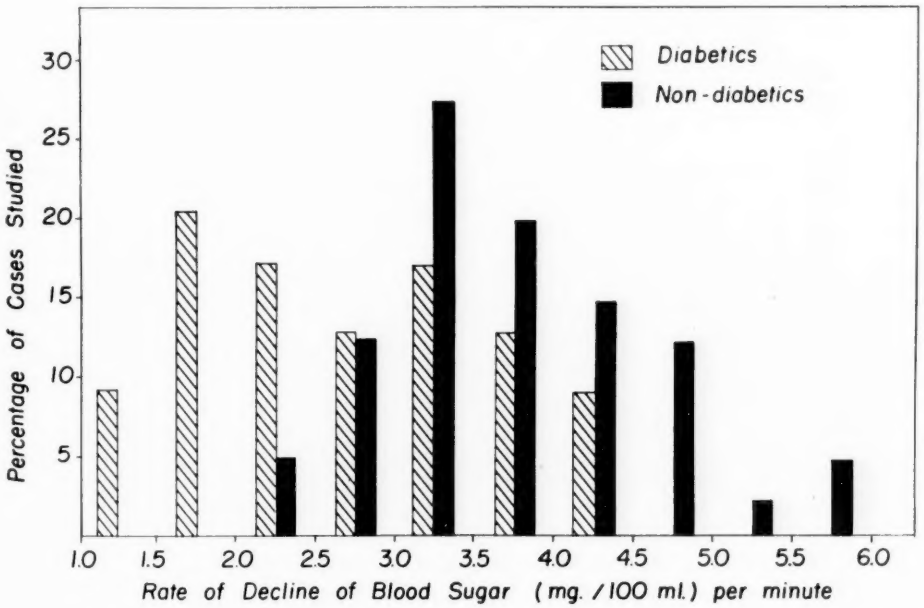


Fig. 2.—Diagram showing the percentage distribution of the rate of decline of the blood sugar level for the thirty-minute interval after insulin administration in the modified G.I.T.T (forty nondiabetic and twenty-four diabetic individuals).

When the ratio of the thirty-minute blood sugar level of the modified G.I.T.T. to the rate of decline was calculated a figure was obtained which we have termed the "sensitivity factor," and which shows with two exceptions (Patients 13 and 21) a general correlation with the patients' daily insulin requirement (Table I). The reasons for the discrepancy in these two cases are not understood.

DISCUSSION

The objection to Himsworth's G.I.T.T. for the detection of insulin sensitivity, namely, that normal individuals do not give a homogeneous pattern of response, seems to have been overcome by the use of the modified procedure. Two factors may account for this advantage: (1) the intravenous administration of both the glucose and insulin eliminates aberrations due to differing rates of intestinal absorption of glucose, and (2) the body mechanisms for hyperglycemia responsiveness which are stimulated by the administration of glucose are set in motion before the insulin is injected, so that the added insulin acts in conjunction with these mechanisms.

TABLE I. DATA ON TWENTY-FOUR DIABETIC PATIENTS ON WHOM THE MODIFIED G.I.T.T. WAS PERFORMED

PATIENT	AGE	SEX	ASSOCIATED CLINICAL CONDITIONS	FASTING BLOOD SUGAR*	BLOOD SUGAR 30 MIN. AFTER GLUCOSE ADMINISTRATION*	RATE OF DECLINE OF BLOOD SUGAR†	SENSITIVITY FACTOR‡	DAILY INSULIN REQUIREMENT (UNITS)	SEVERITY OF DIABETES (GRADE)§
1	80	M	Arteriosclerotic hypertensive heart disease	145	256	4.01	64	0	1
2	58	F	Generalized arteriosclerosis, Parkinson's disease, duodenal ulcer	170	274	4.43	64	10 P.Z.I.	2
3	67	F	Generalized arteriosclerosis, thyrotoxicosis (B.M.R.: +25).	148	294	4.00	73	10 P.Z.I.	2
4	71	F	Arteriosclerotic hypertensive cardiovascular disease, thyrotoxicosis (B.M.R.: +30)	146	270	3.70	73	0	1
5	71	M	Hypertensive cardiovascular disease, generalized arteriosclerosis	148	235	3.20	74	0	1
6	67	M	Generalized arteriosclerosis, hemiplegia	150	290	3.94	74	0	1
7	65	F	Generalized arteriosclerosis, hypertensive cardiovascular disease	145	250	3.00	83	0	1
8	86	M	Generalized arteriosclerosis	145	256	2.87	89	10 P.Z.I.	2
9	80	M	Generalized arteriosclerosis	290	420	4.00	105	15 P.Z.I.	2
10	70	F	Hypertensive cardiovascular disease, generalized arteriosclerosis	220	347	3.23	108	25 P.Z.I.	2
11	66	F	Arteriosclerosis, chronic rheumatic heart disease	230	380	3.33	114	20 P.Z.I.	2
12	46	F	Postencephalitic Parkinsonism	177	335	2.90	116	15 P.Z.I.	2
13	72	F	Arteriosclerotic hypertensive heart disease, nephrosclerosis	145	240	1.93	124	0	1
14	68	M	Carcinoma of rectum, generalized arteriosclerosis	142	240	1.94	124	30 P.Z.I.	2
15	67	M	Generalized arteriosclerosis	162	292	2.33	125	25 P.Z.I.	2

*In mg. per 100 milliliters.
†In mg. per 100 milliliters per minute.
‡Thirty min. blood sugar level/rate of decline.
§Graded according to Wilder.[17]

16	61	M	Arteriosclerotic hypertensive cardiovascular disease, nephrosclerosis, latent syphilis	150	300	2.20	136	35 P.Z.I.	3
17	23	F	Diffuse sclerosis of central nervous system	155	325	2.37	137	40 Reg. + 15 P.Z.I.	3
18	65	F	Arteriosclerotic heart disease, nephrosclerosis	320	480	3.34	143	25 Reg. + 10 P.Z.I.	3
19	51	F	Arteriosclerotic hypertensive heart disease	155	292	1.80	162	30 Reg. + 20 P.Z.I.	3
20	62	F	Arteriosclerotic heart disease, wet gangrene of right lower extremity	200	320	1.84	170	50 Reg. + 30 P.Z.I.	4
21	70	F	Rheumatoid arthritis with hepatitis after chrysotherapy	147	260	1.47	174	20 P.Z.I.	2
22	57	F	Arteriosclerotic heart disease, wet gangrene of left lower extremity	286	405	2.17	186	45 Reg. + 20 P.Z.I.	4
23	68	F	Hypertensive cardiovascular disease, generalized arteriosclerosis	240	385	2.00	193	40 Reg. + 25 P.Z.I.	4
24	27	M	Diffuse sclerosis of central nervous system	270	365	1.34	272	40 Reg. + 45 P.Z.I.	4

It is suggested that the criterion for normal insulin sensitivity using the modified G.I.T.T. be a return of the blood sugar to the fasting level within seventy-five minutes after the glucose administration. This was observed in ten (40 per cent) of the diabetic patients. A return of the blood sugar to the fasting level within seventy-five to ninety minutes as noted in seven (30 per cent) is considered to be an equivocal response. The failure of the blood sugar to return to the fasting value until ninety minutes or longer is interpreted to be evidence of a decreased sensitivity of the individual to the blood sugar lowering activity of insulin. The remaining seven (30 per cent) of the diabetic patients would fall into this category.

The nadir of the blood sugar level is not considered to be significant for insulin sensitivity, since it is a reflection of the point at which the body counter-regulatory mechanisms to the falling blood sugar level become effective.

As previously indicated, the degree of insulin sensitivity can be stated mathematically as the rate of decline of the blood sugar concentration expressed in milligrams per 100 ml. per minute calculated for the period of thirty minutes after the insulin injection. This time interval was utilized since the nondiabetic individual showed a constant rate of decline for this period, and at its end the blood sugar level was not low enough to stimulate counter-regulation.

The rates of decline for the nondiabetic individuals are normally distributed, whereas the diabetic patients show a nonhomogeneous distribution suggestive of two groups (Fig. 2). The group of seven patients (29 per cent) showing values of 2.0 mg. per 100 ml. per minute or less are considered to be insulin insensitive, while the 13 (54 per cent) showing a rate of decline of greater than 2.5 mg. per 100 ml. per minute are interpreted as having normal insulin sensitivity. Since only two (5 per cent) normal individuals had rates of decline of 2.0 to 2.5 mg. per 100 ml. per minute, these values may be considered equivocal as to insulin sensitivity, and the four (17 per cent) diabetic patients who fell into this range cannot be assigned to either group.

Using the two different criteria for insulin sensitivity—namely, the time interval for return of the blood sugar to the fasting level and the rate of decline of the blood sugar—a discrepancy in the results becomes apparent. With the latter criterion only four cases are considered to be equivocal while with the former, seven cases have to be considered in this group. It is believed, therefore, that the rate of decline of the blood sugar is a more exact method for the differentiation of insulin sensitivity from insulin insensitivity.

The associated clinical conditions as a whole did not seem to be connected with the rate of decline of the blood sugar level, both groups having primarily degenerative vascular disorders (Table I). In five patients, however, the concomitant diseases appeared to be factors in the type of response obtained. One of the two patients with wet gangrene of the lower extremity had a definitely impaired sensitivity with a rate of decline of 1.84 mg. per minute; the other had an equivocal response of 2.17 mg. per minute. This lowered insulin sensitivity in the two diabetic patients of our series with infection is in accord with general clinical experience. However, contrary to expectation, the two patients with hyperthyroidism showed normal insulin sensitivity. The clinical

insulin resistance of patients with hyperthyroidism and diabetes might be explained by the fact that increase in thyroid function increases the rate of intestinal absorption of glucose, thus aggravating the diabetes, even though the rate of peripheral glucose utilization will also tend to be increased by the elevation of thyroid function.[12] The patient with hepatitis after chrysotherapy which antedated the onset of diabetes showed a markedly diminished insulin sensitivity which may be explained as a failure of the liver to respond rapidly to added insulin by reducing its glucose output.

It would have been of interest to be able to study the rate of decline in diabetic patients with pituitary or adrenal hyperfunction or deficiency; however, these were not available.

As an incidental finding in this study, it was noted in several nondiabetic individuals that the blood sugar dropped to 18 to 25 mg. per cent, which is below the level generally considered to be critical for the development of a hypoglycemic reaction[13] without their showing any of the symptomatology or only very minor symptoms of hypoglycemia. This would be in keeping with a previous hypothesis that the onset of a hypoglycemic reaction may be determined by the concentration of glucose in the tissues rather than in the blood.[14]

It may seem, theoretically, that the difference between the rate of decline of the blood sugar level for this thirty-minute interval of the G.T.T. and that of the modified G.I.T.T. would be a more accurate index of insulin sensitivity than the rate of decline in the modified G.I.T.T. alone. However, as evidenced by a comparison of Figs. 1*A* and 1*C*, the difference between the rate of decline of the blood sugar level in the G.T.T. and modified G.I.T.T. for this period is frequently less in normal individuals than that observed in some diabetic patients. This would imply that these diabetic individuals have a greater insulin sensitivity than the normal individual which is not borne out by the rate of decline of the blood sugar level as observed in the modified G.I.T.T. alone.

The question may also be raised as to whether the degree of insulin sensitivity of an individual as obtained with the modified G.I.T.T. is predicated on the particular dosage of insulin used. This objection is, however, on theoretical grounds not probable, since it has been shown that the effect of insulin on the blood sugar level is not a stoichiometric relationship. It has been demonstrated, for example, that 6 units of insulin injected intravenously in a normal individual increased the rate of decline of the blood sugar level only very minutely over the rate of decline after 3 units of insulin.[2] The explanation for this phenomenon is that the rate of entry of glucose into the tissues and liver probably approaches a maximum, even with small insulin doses.

It is interesting in this connection to note that if the mean rate of glucose decline for the normal individual under the influence of insulin is recalculated in terms of the rate of disappearance of glucose from the blood stream, assuming a 70 kilogram individual with an extracellular fluid compartment of 14 liters, a value of 480 mg. per kilogram per hour is obtained, which corresponds quite well with the values obtained by Soskin and Levine[15] in the dog for the maximal rate of glucose uptake by the peripheral tissues, i.e., 550 mg. per kilogram per hour. This would seem to indicate that for the thirty-minute period of the

modified G.I.T.T. utilized, the chief site of glucose assimilation is the peripheral tissue rather than the liver. This is further supported by the work of Bondy and associates[16] who demonstrated that although the glucose output from the liver starts to decline almost immediately after insulin administration, the liver does not start to take up glucose for approximately forty-five minutes.

On the other hand, the use of large nonphysiologic doses of insulin in this procedure would probably overwhelm the mechanisms involved and thus blur the significance of the results as to the sensitivity of the individual patient.

The rate of decline of the blood sugar level from the thirty- to sixty-minute specimens of the modified G.I.T.T. is therefore a figure which is an indicator for the rate of disappearance of glucose from the extracellular compartment in the presence of insulin, and is thus a measure of insulin sensitivity. The diabetic patients who show a rate of decline of the blood sugar after insulin less than that observed in the normal are considered to be insulin insensitive in the true sense of the word, i.e., the rate of glucose disappearance from the blood stream under the influence of a standard insulin dose is less than that of a normal person. These patients may have excess insulin antagonistic factors which interfere with the effectiveness of insulin in increasing peripheral glucose assimilation or with the action of insulin in inhibiting the hepatic release of glucose. Or they may have an intrinsic hepatic dysfunction as to carbohydrate metabolism, so that the liver cannot respond to administered insulin by decreasing its glucose output.

The validity of the rate of decline as a measure of insulin sensitivity appears to be borne out clinically. Since in spite of the fact that neither the fasting nor the thirty-minute blood sugar level nor the rate of decline as such showed any correlation with the patients' daily insulin requirements, the "sensitivity factor," i.e., the ratio of the thirty-minute level to the rate of decline, appears to show a definite general relationship (Table I).

SUMMARY

A modification of Himsworth's Glucose Insulin Tolerance Test is presented which consists of the administration of 0.1 unit of crystalline insulin intravenously thirty minutes after the intravenous injection of 25 Gm. of glucose in 50 per cent solution. This procedure elicited a homogeneous pattern of response in forty nondiabetic individuals, the blood sugar falling to the fasting level within forty-five minutes after the administration of insulin. Using this time interval for the return of the blood sugar to the fasting level as the criterion for normal insulin sensitivity ten of the diabetic patients showed a normal response, while seven patients showed evidence of resistance and seven gave an equivocal response.

As an alternative and more exact criterion for insulin sensitivity, the rate of decline of the blood sugar level for the thirty minutes after injection of insulin may be used. The mean value for the nondiabetic controls is 3.8 mg. per 100 ml. per minute, with a standard deviation of 0.7 mg. per 100 ml. per minute. Of the diabetic patients, seven (29 per cent) had values of less than 2 mg. per 100 ml. per minute, and thus are considered to be insulin in-

sensitive. Four patients (17 per cent) showed an equivocal response ranging from 2.0 to 2.4 mg. per 100 ml. per minute, and the remaining thirteen patients (54 per cent) had values of 2.5 to 5.0 mg. per 100 ml. per minute, and were considered to be normally sensitive to the action of insulin. The nonhomogeneous distribution of the results obtained with this procedure in the diabetic patients tends to support their differentiation into an insulin-sensitive and insulin-insensitive group.

The ratio of the thirty-minute blood sugar level to the rate of decline which was termed the "sensitivity factor" showed, in general, a correlation with the daily insulin requirement of the patient.

The authors wish to express their grateful appreciation to Dr. Co Tui for his helpful criticism and advice during the preparation of this paper.

REFERENCES

1. Himsworth, H. P.: Diabetes Mellitus; Its Differentiation into Insulin-Sensitive and Insulin-Insensitive Types, Lancet **1**: 127-130, 1936.
2. Somogyi, M.: Studies of Arteriovenous Differences in Blood Sugar. III. Effect of Insulin Administered Intravenously in the Postabsorptive State, J. Biol. Chem. **179**: 217-233, 1949.
3. Himsworth, H. P., and Kerr, R. B.: Insulin-Sensitive and Insulin-Insensitive Types of Diabetes Mellitus, Clin. Sc. **4**: 119-155, 1939.
4. DeWesselow, O. L. V., and Griffiths, W. J.: Insulin Resistance in Diabetes Mellitus and the Effect of Dietary Carbohydrate, Quart. J. Med. **7**: 17-28, 1938.
5. Fraser, R., Albright, F., and Smith, P. H.: The Value of the Glucose Tolerance Test, the Insulin Tolerance Test, and the Glucose-Insulin Tolerance Test in the Diagnosis of Endocrinologic Disorders of Glucose Metabolism, J. Clin. Endocrinol. **1**: 297-306, 1941.
6. Soskin, S., and Levine, R.: Experimental Insulin-Sensitive and Insulin-Insensitive Diabetes, J. A. M. A. **110**: 768, 1938.
7. Leevy, C. M., Ryan, C. M., and Fineberg, J. C.: Diabetes Mellitus and Liver Function, Am. J. Med. **8**: 290-299, 1949.
8. Zimmerman, H. J., MacMurray, F. G., Rappaport, H., and Alpert, L. K.: Studies of the Liver in Diabetes Mellitus. II. The Significance of Fatty Metamorphosis and Its Correlation With Insulin Sensitivity, J. Lab. & Clin. Med. **36**: 922-928, 1950.
9. Kirketerp, P.: The Insulin-Sensitivity of Patients With Diabetes Mellitus, J. Clin. Investigation **29**: 1633-1635, 1950.
10. Nelson, N.: Photometric Adaptation of Somogyi Method for Determination of Glucose, J. Biol. Chem. **153**: 375-380, 1944.
11. Lozner, E. L., Winkler, A. W., Taylor, F. H. L., and Peters, J. P.: Intravenous Glucose Tolerance Test, J. Clin. Investigation **20**: 507-515, 1941.
12. Soffer, L. J.: Diseases of the Endocrine Glands, Philadelphia, 1951, Lea and Febiger, p. 725.
13. Duncan, G. G.: Diabetes Mellitus. Principles and Treatment, Philadelphia, 1951, W. B. Saunders Co., p. 154,
14. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry. Interpretations, Vol. 1, Baltimore, 1946, The Williams and Wilkins Co., pp. 228-229.
15. Soskin, S., and Levine, R.: Carbohydrate Metabolism, Chicago, 1946, University of Chicago Press, p. 183.
16. Bondy, P. K., Bloom, W. L., Whitner, V. S., and Farrar, B. W.: Studies of the Role of the Liver in Human Carbohydrate Metabolism by Venous Catheter Technic. II. Patients With Diabetic Ketosis, Before and After the Administration of Insulin, J. Clin. Investigation **28**: 1126-1133, 1949.
17. Wilder, R. M.: Clinical Diabetes Mellitus and Hyperinsulinism, Philadelphia, 1941, W. B. Saunders Co., p. 59.

THE DISAPPEARANCE OF INTRAVENOUSLY INJECTED EMULSIFIED FAT FROM THE CIRCULATION OF PATIENTS AND ANIMALS

WILLIAM A. JOHNSON, M.D., SMITH FREEMAN, M.D., PH.D., AND KARL A. MEYER, M.D.
CHICAGO, ILL.

IN RECENT years considerable evidence has accumulated indicating that parenterally administered fat can be utilized by animals[1-9] and man.[10-13] However, characteristics of removal of injected fat from the circulation have received scant attention, although this is a part of the response of the organism to the injection.

By comparison of the removal of parenterally administered fat in normal subjects and in patients with metabolic disturbances that may involve fat transport or utilization, one may gain some evidence as to whether or not the injected fat is treated as a foreign substance or similarly to absorbed fat. The following experiments deal with this subject. These studies were conducted on human subjects with minor complaints that were amenable to surgery and on patients with renal insufficiency, hyperthyroidism, and cirrhosis of the liver. Similar studies were conducted on dogs before and after ureteral ligation, before and after Eck fistula formation, and in controlled and uncontrolled alloxan diabetes.

PROCEDURE

The fat emulsion used in these studies contained 10 per cent olive oil in 5 per cent glucose emulsified with a 1 per cent lecithin fraction* in the manner previously described.[14] Analysis was made for total serum fatty acids,[15] cholesterol,[16] and lipid phosphorus[17] before, during, and after injection of the fat emulsion.

As a preliminary experiment two patients were given 1 gram of fat per kilogram body weight at different injection rates (two and four hours) on different days; and on another day the same volume of 1 per cent lecithin in 5 per cent glucose was administered over a four-hour period of time. In each instance blood was drawn after twelve hours of fast, again at the end of injection and two, four, and eight hours after completion of the injection.

Next a group of nine patients hospitalized for nonmetabolic complaints, such as hemorrhoids, were given 1 gram per kilogram of emulsified fat over a four-hour period, and the disappearance of injected fat was determined. In each instance blood was drawn before, in the middle, at the end, and two, four, and eight hours after completion of the fat injection. Similar studies were made on five patients with characteristic clinical manifestations of cirrhosis of the liver, six patients with toxic manifestations of hyperthyroidism, and on two patients with advanced glomerulonephritis with marked elevation of the nonprotein nitrogen.

Similar studies were made upon the dogs except that 0.5 Gm. of fat per kilogram was injected over a one-hour period. In every instance repeated injections on separate days were done in each animal to verify the reproducibility of the results. Two dogs were studied before and after Eck fistula formation. Rose bengal dye clearance was used to estimate the reduction in hepatic function following the shunt operation. Two dogs were studied before and twenty-four and seventy-two hours after bilateral ureteral ligation. One animal

From the Department of Experimental Medicine, Northwestern University Medical School, and Cook County Hospital.

*The lecithin was kindly supplied through the courtesy of Dr. Percy Julian of Glidden and Company, Chicago, Ill.

Received for publication, Oct. 12, 1951.

also received a fat injection ninety-six hours after ureteral ligation. Two alloxan diabetic dogs with moderately severe diabetes, controlled by insulin so that the urine contained less than 5 Gm. of sugar daily, received fat injections on two different days. Thereafter insulin was discontinued for three days and the glycosuria, blood sugar, and disappearance of injected fat were determined. The animals were again brought under control with insulin and the whole process was then repeated.

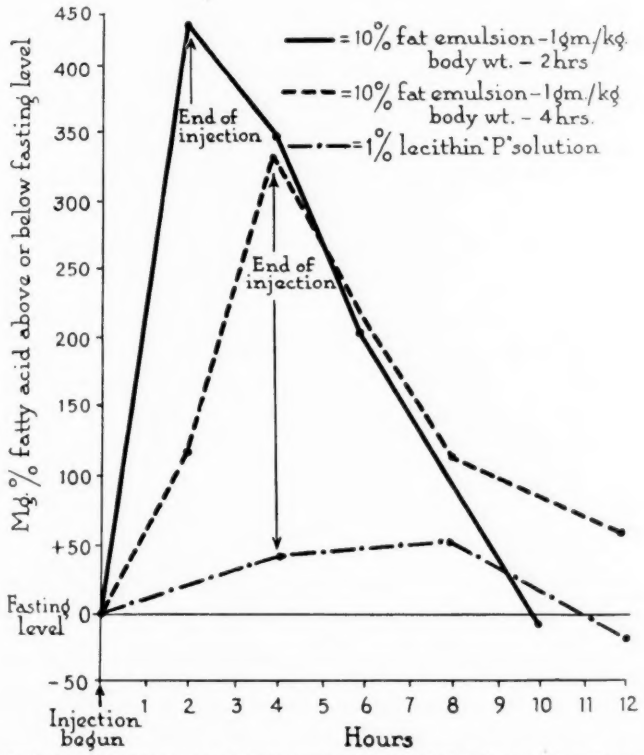


Fig. 1.—Rate of disappearance of intravenously injected fat emulsion from the circulation of a human subject.

RESULTS

The preliminary studies indicated that the time of return to the fasting level was similar when the fat injection was given in two or four hours, and that the emulsifying agent in glucose caused only a slight rise in the lipid content of the plasma (see Fig. 1). If one assumes a blood volume of 9 per cent, and calculates from the plasma volume and fat concentration the amount of injected fat circulating at the end of injection, and subtracts this value from the total amount injected, one finds that 74.6 per cent of the injected fat had been removed by the end of the injection in the nine normal patients. In the thyrotoxic and cirrhotic patients the proportion of injected fat removed by the end of the injection was 87.4 per cent and 85.6 per cent, respectively. The most striking difference was noted in the glomerulonephritic patients in whom only 37 per cent of the injected fat had been removed by the end of the injection. From Table I it can be seen that the cirrhotic and thyrotoxic patients had less fatty acid rise than the normal group, while the two patients with glomerulo-

nephritis had a markedly elevated fatty acid level at the end of the infusion. These data suggest that a metabolic disturbance may influence the rate of removal of parenterally infused fat in human subjects.

Parenteral infusion of fat caused an initial rise in plasma cholesterol followed by a decline in every patient except those with cirrhosis, in whom the serum cholesterol values decreased during the infusion (see Table I). A decrease below the initial values was observed eight hours after completion of fat infusion on all subjects except those with glomerulonephritis.

The behavior of lipid phosphorus was quite similar to that of the fatty acids and cholesterol (see Table I) in that the patients with glomerulonephritis had the greatest and most prolonged elevation of lipid phosphorus.

TABLE I. DISAPPEARANCE OF PARENTERAL FAT FROM THE CIRCULATION OF PATIENTS*

GROUP OF PATIENTS	NO. OF PATIENTS	DETERMINATION**	FASTING	AT END OF INJECTION	FOUR HOURS AFTER	EIGHT HOURS AFTER
Normal†	9	Total fatty acids	613 ± 201	1123 ± 204	734 ± 245	669 ± 219
		Cholesterol	219 ± 51.7	236 ± 60.4	214 ± 33.6	206 ± 40.5
		Phospholipids	8.62 ± 2.13	10.95 ± 1.96	10.11 ± 1.92	9.29 ± 2.23
Cirrhotic‡	5	Total fatty acids	511 ± 93.6	781 ± 179	612 ± 99.4	524 ± 97.7
		Cholesterol	176 ± 68.1	166 ± 67.4	125 ± 21.8§	149 ± 76.0
		Phospholipids	8.4 ± 2.73	8.5 ± 2.02	7.7 ± 2.38	7.3 ± 2.16
Thyrotoxic‖	6	Total fatty acids	548 ± 178	835 ± 216	559 ± 94.5	468 ± 69.8§
		Cholesterol	209 ± 37.4	217 ± 37.0	198 ± 30.6	191 ± 20.4§
		Phospholipids	9.1 ± 0.92	10.2 ± 1.28	9.2 ± 1.36	8.7 ± 0.16
Glomerulonephritic¶	2	Total fatty acids	486 ± 81.4	1517 ± 292	778§	497§
		Cholesterol	158 ± 14.1	174 ± 14.1	172§	168§
		Phospholipids	8.99 ± 1.42	11.0 ± 2.41	8.99§	8.65§

*1 gram of fat per kilogram body weight of a 10 per cent fat emulsion given in four hours.

**Determinations are in mg. per cent ± the standard deviation.

†Hospitalized for hemorrhoids, etc.

‡Leg edema and ascites present in all; icterus value range 40 to 89 units; cephalin flocculation 3+ to 4+.

§Number of samples in these cases was one less than that indicated in the second column.

‖BMR range +44 to +75; all presented clinical toxicity.

¶N. P. N. range 154 to 252 mg. per cent; creatinine range 12 to 13.2 mg. per cent; hypertension present.

The experiments on the dogs also indicated that metabolic disturbances may influence the rate of removal of infused fat (see Table II). The Eck fistula animals showed the least elevation of the plasma fatty acids two hours after completion of fat infusion, the elevation being less than that on the normal animal. The alloxan diabetic dogs showed considerably more fatty acid elevation than had the normal or diabetic dogs receiving insulin. The behavior of cholesterol differed considerably in the different groups. The controlled and uncontrolled diabetic animals showed the greatest increase and decline of cholesterol, while the Eck fistula dogs showed the least elevation and decline. The latter resembles the cirrhotic patients who showed no elevation but a

TABLE II. DISAPPEARANCE OF PARENTERAL FAT FROM THE CIRCULATION OF DOGS*

	NO. OF EXPERIMENTS	DETERMINATIONS§	FASTING	AT END OF INJECTION	TWO HOURS AFTER	FOUR HOURS AFTER
Normal	4	Total fatty acids	543 ± 86.0	742 ± 54.4	526 ± 89.6	481 ± 77.0
		Cholesterol	163 ± 3.46	183 ± 11.9	169 ± 6.64	161 ± 8.06
		Phospholipids	12.6 ± 0.2	13.9 ± 0.02	12.8 ± 0.46	12.3 ± 0.32
After Eck fistula formation	4	Total fatty acids	333 ± 104	529 ± 175	357 ± 87.2	320 ± 60.6
		Cholesterol	104 ± 9.8	117 ± 12.8	109 ± 17.4	100 ± 13.9
		Phospholipids	7.4 ± 0.88	8.0 ± 0.78	7.3 ± 0.85	6.8 ± 0.88
Normal	4	Total fatty acids	556 ± 75.1	868 ± 176	557 ± 77.2	522 ± 69.8
		Cholesterol	197 ± 35.9	212 ± 28.6	203 ± 33.8	197 ± 314
		Phospholipids	14.9 ± 1.9	16.4 ± 2.45	15.1 ± 2.45	14.5 ± 2.48
After† bilateral ureteral ligation	4	Total fatty acids	567 ± 19.1	876 ± 28.6	607 ± 22.8	574 ± 15.5
		Cholesterol	248 ± 30.2	263 ± 24.3	244 ± 18.0	239 ± 22.3
		Phospholipids	16.3 ± 3.46	18.1 ± 4.01	15.2 ± 2.81	14.9 ± 2.24
Controlled diabetes	4	Total fatty acids	903 ± 90.8	1182 ± 64.7	907 ± 52.0	757 ± 83.3
		Cholesterol	298 ± 24.9	334 ± 16.5	314 ± 1.99	295 ± 9.44
		Phospholipids	19.4 ± 0.44	20.4 ± 0.39	19.5 ± 0.43	18.8 ± 0.56
Uncontrolled diabetes‡	4	Total fatty acids	1696 ± 344	2155 ± 120	1759 ± 374	1546 ± 244
		Cholesterol	312 ± 105	346 ± 36.6	319 ± 22.0	304 ± 26.7
		Phospholipids	21.8 ± 2.94	23.9 ± 2.39	22.2 ± 1.98	20.4 ± 1.31

*0.5 gram per killogram body weight of a 10 per cent fat emulsion given in one hour.
†Twenty-four and seventy-two hours after ureteral ligation.
‡183 Gm. glucose in urine per twenty-four hours.
§Determinations are in mg. per cent ± the standard deviation.

definite decline in plasma cholesterol. The increase in phospholipids was similar to that of the fatty acids and had largely returned to normal two hours after completion of the fat infusion in all groups of dogs.

DISCUSSION

Seventy-five per cent of injected fat was removed from circulation in normal subjects by the end of the infusion, whereas 8 hours longer were required to remove the remainder.

Alterations in fat transport and metabolism in liver disease might be expected since this organ plays an important part in phospholipid[18, 19] and cholesterol ester formation,[20, 21] as well as in the desaturation[22, 23] and oxidation of the fatty acids.[24, 25] Abnormalities in the lipid content of the blood have been reported to occur in patients with liver disease. In general, the literature indicates that a hypolipemia is usually associated with parenchymal damage to the liver.[26] This is also true in the Eck fistula dog.[27] That this hypolipemia may be related to an increased rate of removal of fat from the circulation is suggested by the observations on cirrhotic patients and Eck fistula dogs. The results of the present study both on patients and animals suggest that impairment of hepatic function accelerates fat removal from the circulation.

It has been previously shown that, in general, cholesterol (and similarly neutral fat and phospholipids) decrease in hyperthyroidism and increase in

myxedema.[28, 29] The present study on thyrotoxic patients indicates that there is a more rapid removal of injected fat from the circulation than in normal persons.

There is considerable clinical[30, 31] and some experimental[32, 34, 35, 36] evidence that renal damage may alter the fat content of the blood stream. The increased fat content so characteristic of lipoid nephrosis has been ascribed to the hypoproteinemia.[33] Lipemia has been reported to occur following nephrectomy and ureteral ligation in animals[32, 34, 35] and also following experimental damage to the kidneys by injection of hypertonic sucrose[36] and other reagents.[34] The mechanism of this lipemia is not known. The results observed on renal insufficient patients and ureteral ligated dogs injected with fat emulsion suggest that lipemia associated with renal disease may at least be due in part to a decreased rate of removal of fat from the circulation.

The lipemia of uncontrolled diabetes is thought to be due to increased fat mobilization,[37, 38] although it has also been suggested that there is a reduced ability to remove fat from the circulation in diabetes mellitus.[39] Presumably the turnover of fat is unusually high in the diabetic organism deprived of insulin. The high plasma lipid concentration after fat infusion and rapid rate of plasma fat removal observed in the uncontrolled diabetic dogs is in accord with the above explanation.

SUMMARY

1. The rate of removal of injected fat from the blood stream in normal persons is influenced by the concentration of blood fat.

2. Patients with glomerulonephritis and dogs with bilateral ureteral ligations were found to remove fat more slowly from the blood stream than the normal groups.

3. There was less elevation of the blood fat of cirrhotic patients and Eck fistula dogs following the administration of parenteral fat than for their controls.

4. Persons with thyrotoxicosis removed infused fat more rapidly than the control group.

5. Removal of injected fat from the circulation of uncontrolled diabetic dogs was slower than that of the insulin control periods for the same animals, but the total decline in serum fatty acids during the period of observation was greater for both controlled and uncontrolled diabetic dogs than for any other group.

REFERENCES

1. Murlin, J. R., and Riche, J. A.: Blood Fat in Relation to Heat Production and Depth of Narcosis, Proc. Soc. Exper. Biol. & Med. **13**: 7-8, 1915.
2. Baba, T.: Über den Abbau direkt infundierten Fetts im Tierkörper; Grundumsatz pankreasdiabetischer Tiere nach Fettinfusion, Tohohu J. Exper. Med. **17**: 274-292, 1931.
3. Dunham, L. J., and Brunschwig, A.: Intravenous Administration of Fat for Nutritional Purposes, Arch. Surg. **48**: 395-405, 1944.
4. Narat, J. K.: Observations on Parenteral Administration of Fat Emulsions, Am. J. Digest. Dis. & Nutrition **4**: 107-109, 1937.
5. McKibbin, J. M., Ferry, R. M., Jr., and Stare, F. J.: The Utilization of Emulsified Fat Given Intravenously, J. Clin. Investigation **25**: 679-686, 1946.

6. Meng, H. C., and Freeman, S.: Experimental Studies on the Intravenous Injection of a Fat Emulsion Into Dogs, J. LAB. & CLIN. MED. **33**: 689-707, 1948.
7. Geyer, R. P., Chipman, J., and Stare, F. J.: Oxidation *in Vivo* of Emulsified Radioactive Trilaurin Administered Intravenously, J. Biol. Chem. **176**: 1469, 1948.
8. Lerner, S. R., Chaikoff, I. L., Entenman, C., and Dauben, W. G.: Oxidation of Parenterally Administered C^{14}-labeled Tripalmitin Emulsions, Science **109**: 13, 1949.
9. Meng, H. C.: A Fat Emulsion for Intravenous Nutrition in Rabbits, J. LAB. & CLIN. MED. **37**: 222-28, 1951.
10. Gordon, H. H., and Levine, S. Z.: Effect of Intravenous Infusions of Fat on the Energy Exchange of Infants, Am. J. Dis. Child. **50**: 894-912, 1935.
11. Helfrick, F. W., and Abelson, N. M.: Intravenous Feeding of Complete Diet in a Child, J. Pediat. **25**: 400-403, 1944.
12. Holt, L. E., Tidwell, H. C., and Scott, T. F. M.: The Intravenous Administration of Fat, J. Pediat. **6**: 151-160, 1935.
13. Clark, D. E., and Brunschwig, A.: Intravenous Nourishment With Protein, Carbohydrate, and Fat in Man, Proc. Soc. Exper. Biol. & Med. **49**: 329-332, 1942.
14. Johnson, W. A., and Freeman, S.: Some Effects of Intravenous Fat Emulsions on Human Subjects, J. LAB. & CLIN. MED. Feb. issue.
15. Smith, M. E., and Kik, M. C.: A Micromethod for the Determination of Fatty Acids from Small Amounts of Whole Blood, J. Biol. Chem. **103**: 391-398, 1933.
16. Hoffman, W.: Serum Cholesterol Determination, Photelometric Clinical Chemistry, New York, 1941, William Morrow and Company, p. 124.
17. Gomori, G.: A Modification of the Colorimetric Phosphorus Determination for Use With the Photoelectric Colorimeter, J. LAB. & CLIN. MED. **27**: 955-960, 1942.
18. Cavenagh, B., and Raper, H. S.: A Study of the Passage of Fatty Acids of Food into Lipins and Glycerides of the Body, Using Deuterium as an Indicator, Biochem. J. **33**: 17-21, 1939.
19. Sinclair, R. G.: The Metabolism of the Phospholipids. I. The Influence of Diet on the Amount and Composition of the Phospholipid Fatty Acids in Various Tissues of the Cat, J. Biol. Chem. **86**: 579-586, 1930.
20. Gardener, J. A., and Gainsborough, A.: Blood Cholesterol Studies in Biliary and Hepatic Disease, Quart. J. Med. **23**: 465-483, 1930.
21. Hawkins, W. B., and Wright, A.: Blood Plasma Cholesterol: Fluctuations Due to Liver Injury and Bile Duct Obstruction, J. Exper. Med. **59**: 427-439, 1934.
22. Rittenberg, D., and Schoenheimer, R.: Deuterium as an Indicator in the Study of Intermediary Metabolism. Hydrogenation of Fatty Acids in Animal Organism, J. Biol. Chem. **117**: 485-490, 1937.
23. Hilditch, T. P.: The Chemical Constitution of Natural Fats, New York, 1941, John Wiley & Sons, Inc., p. 276.
24. Butts, J. S., Cutler, C. H., Hallman, L., and Deuel, H. J. Jr.: Quantitative Studies on Beta-oxidation, J. Biol. Chem. **109**: 597-613, 1935.
25. Jowett, M., and Quastel, J. H.: The Oxidation of Normal Saturated Fatty Acids in the Presence of Liver Slices, Biochem. J. **29**: 2159-2180, 1935.
26. Man, E. B., Kartin, B. L., Durlacher, S. H., and Peters, J. P.: Lipids of Serum and Liver in Patients With Hepatic Diseases, J. Clin. Investigation **24**: 623-643, 1945.
27. Winter, I. C., Van Dolah, J. E., and Crandall, L. A., Jr.: Lowered Serum Lipid Levels in the Eck Fistula Dog, Am. J. Physiol. **133**: 566-571, 1941.
28. Peters, J. P., and Man, E. B.: The Interrelations of Serum Lipids in Patients With Thyroid Disease, J. Clin. Investigation **22**: 715-720, 1943.
29. Man, E. B., Gildea, E. F., and Peters, J. P.: Serum Lipoids and Proteins in Hyperthyroidism, J. Clin. Investigation **19**: 43-59, 1940.
30. Peters, J. P., and Man, E. B.: The Interrelations of Serum Lipids in Patients With Diseases of the Kidneys, J. Clin. Investigation **22**: 721-726, 1943.
31. Hiller, A., Linder, G. C., Lundsgaard, C., and Van Slyke, D. D.: Fat Metabolism in Nephritis, J. Exper. Med. **39**: 931-955, 1924.
32. Winkler, A. W., Durlacher, S. H., Hoff, H. E., and Man, E. B.: Changes in Lipid Content of Serum and of Liver Following Bilateral Renal Ablation or Ureteral Ligation, J. Exper. Med. **77**: 473-486, 1943.
33. Fishberg, E. H., and Fishberg, A. M.: The Mechanism of the Lipemia of Bleeding, Proc. Soc. Exper. Biol. & Med. **25**: 296-299, 1927-28.
34. Heymann, W., and Clark, E. C.: Pathogenesis of Nephrotic Hyperlipemia, Am. J. Dis. Child. **70**: 74-82, 1945.
35. Heymann, W., and Sekerak, B.: "Renal Hyperlipemia" in Dogs and Rats, Proc. Soc. Exper. Biol. & Med. **60**: 276-279, 1945.
36. Heymann, W., and Hartman, M. E.: Hyperlipemia Following Intravenous Infusion of Hypertonic Solution of Sucrose, Am. J. Dis. Child. **75**: 68-75, 1948.
37. Bing, H. I., and Heckscher, H.: Uber die Fettmenge des Blutes bei normalen Menschen, Biochem. Z. **149**: 83-89, 1924.
38. Thannhauser, S. J.: Serum Lipids and Their Value in Diagnosis, New England J. Med. **237**: 515, 1947.
39. Bloor, W. R.: Lipemia, J. Biol. Chem. **49**: 201, 1921.

THE EFFECT OF SWEATING ON NORMO- AND HYPERTENSIVE SUBJECTS

THOMAS B. GIBBONS, M.D., AND CARLETON B. CHAPMAN, M.D.
MINNEAPOLIS, MINN.

LOW salt diets are known to produce a decline in blood pressure in some patients with essential hypertension.[1, 2] Whether or not a hypotensive effect is observed, the diets induce a definite loss of body water, particularly from the extracellular compartment. Such diets also produce loss of serum electrolyte. The rice-fruit diet, for example, causes definite lowering of serum chloride and elevation of serum bicarbonate, with little change in the concentration of serum sodium and potassium.[2] Whether the loss of body water or the changes in electrolyte composition, or both, are responsible for the depressor effect such diets may produce is not known.

One possibility, however, is that the depressor effect of salt depletion regimens is a direct result of the loss of body water and that electrolyte depletion, per se, is only indirectly responsible. The induction of loss of body water, with relatively small loss of electrolytes, offers a means of testing the possibility. In order to accomplish this, a group of normal and hypertensive subjects were subjected to sweating in a hot room until significant loss of body water occurred.

MATERIAL

Eight normal men, aged 18 to 27 years, were employed as controls. The average resting blood pressure in the group was 124/66 mm. Hg.

A total of 23 hypertensive men was studied. Of this number, 7 were subjected to the sweating procedure. All members of the group had sustained essential hypertension; the average resting blood pressure was 184/118 mm. Hg, systolic pressure ranging from 148 to 225, and diastolic pressures from 95 to 150 mm. Hg. The ages ranged from 17 to 50 years.

There was no evidence of nitrogen retention or of congestive heart failure in any of the hypertensive subjects. All members of the group were ambulatory and none was limiting his intake of salt at the time of the experiments.

METHOD

For estimation of basal fluid volumes, the subjects reported to the laboratory after fasting for at least fourteen hours. After thirty minutes' rest in bed, three or more sphygmomanometric readings were taken, and a 15 ml. basal blood sample was drawn. The basal sample was centrifuged and inspected for hemolysis. Exactly 20 ml. of a solution containing 13.58 mg. of T-1824 dye, 345 mg. of sodium thiocyanate, and 1.0 Gm. of antipyrine were then injected intravenously in the space of one minute, the midpoint of the injection being taken as zero time. With the subject remaining completely at rest, blood samples were then drawn beginning ten minutes after injection, and ending two hundred forty minutes after it.

Estimates of the plasma volume were carried out by the method of Gibson and Evans,[3] as modified by Gibson and Evelyn.[4]

From the Laboratory of Physiological Hygiene, and the Department of Medicine, University of Minnesota.

This study was supported by a grant from the Minnesota Heart Association.
Received for publication, Nov. 8, 1951.

The thiocyanate dilution method of Crandall and Anderson[5] was used for estimating the extracellular fluid volume. The plasma concentrations of thiocyanate were plotted on semilogarithm paper and two lines were drawn, the first through the 10- and 20-minute values, and the second between the 30-, 40-, and 50-minute values. The point of intersection was taken as the equilibrium point, and usually fell twenty to forty minutes after injection.

Total body water was estimated by the antipyrine method of Soberman and associates.[6] Three blood samples, drawn two, three, and four hours after injection, were employed for the estimation.

The sweating procedure was usually carried out on the subjects' second or third visit to the laboratory, several days after the basal fluid volume determinations. In most instances, control determinations of thiocyanate and T-1824 spaces were carried out on the same individuals on two separate days. The antipyrine space was measured in this manner in five of the subjects. In a few cases it was necessary to carry out the sweating procedure first, basal fluid volumes being measured three to seven days later. Multiple basal blood pressure readings were taken after thirty minutes of rest in bed. The subjects were then weighed nude and were taken into a room heated to 110 to 120° F., with a relative humidity of 60 to 80 per cent. Fluid loss was hastened by frequent periods of exercise on a motor-driven treadmill, usually running at 3 or 3.5 MPH, and set at 2.5 to 5 per cent grade. The periods of exercise were five to ten minutes in length, and were spaced at 30- to 60-minute intervals, depending on the patient's capacity for exercise at high temperature. Measurements of pulse rate, blood pressure, body temperature, and body weight were made at frequent intervals. As was expected, the normotensive subjects tolerated the procedure better than did the hypertensive subjects. Several of the latter experienced such extreme discomfort that the high temperature phase had to be discontinued prematurely. In some instances the subjects could perform only a small amount of exercise so that the desired level of fluid loss was not attained, but in no case did signs of circulatory collapse appear.

The subjects were allowed to take a few ice cubes during the period in the hot environment, but apart from this no food or fluids were provided. In no case did the water consumed in this manner exceed 200 milliliters. When the weight of the subject had declined about 4 per cent, which required from four to eight hours, the subjects were removed from the hot environment and placed at rest in a separate room. When rectal temperatures had returned to normal (thirty or forty minutes later), fluid volume and specific gravity determinations were repeated.

Methodologically, the reliability of the techniques used for determination of fluid spaces is open to question. Of the three methods used, the reliability of the T-1824 technique for estimating plasma volume is least in doubt. It is reported by Gregersen[7] to be accurate within ±1 per cent. The validity and accuracy of the thiocyanate technique, as pointed out by Leavitt and Gaudino,[8] are in doubt because the thiocyanate ion is taken up by certain body cells, especially in various abnormal states. These authors also believe that "the thiocyanate space is not constant in the same animal under identical conditions." The experience with normal human beings in our laboratory is not in accord with this view. Keys and asociates,[9] working with normal young men, found that the mean thiocyanate space was 16.06 liters before semi-starvation (21 men) and 16.35 liters after full recovery from it (13 men) twenty-one months later. Expressed as per cent of total weight, the corresponding figures are 23.64 ± 1.65, and 23.51 ± 1.43. Our own experience with the thiocyanate method in 15 normotensive and hypertensive men yielded mean values of 17.73 ± 2.34 and 17.60 ± 2.48 liters for first and second trials, respectively. Expressed as per cent of total body weight, the corresponding values are 23.95 ± 3.56 and 23.66 ± 2.83. Variance analysis on these data (expressed as liters) discloses that the interindividual component accounts for 75.85 per cent of the total variance, and that the repeatability is quite high. The coefficient of consistency was 0.712, and the standard random error was 1.3 liters. The debate as to the true significance of the results provided by the method is not answerable with certainty, since the absolute size of the extracellular fluid space in normal individuals is unknown. There is good reason to believe, however, that the thiocyanate space is quantitatively, but not absolutely, related to it and, since the repeatability

TABLE I. FLUID SPACES BEFORE AND AFTER SWEATING PROCEDURE

SUBJECTS	ANTIPYRINE SPACE (LITERS)		THIOCYANATE SPACE (LITERS)		ANTIPYRINE MINUS THIOCYANATE SPACE (LITERS)		PLASMA VOLUME (LITERS)		BLOOD VOLUME (LITERS)	
	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
Normotensives										
E. S.	39.08	35.23	16.22	14.48	22.86	20.75	3.16	2.86	5.70	5.40
J. L.	36.05	33.21	18.27	14.18	17.78	19.03	3.25	2.85	5.61	5.39
J. A.	35.08	–	18.04	14.91	17.04	–	3.28	2.48	5.70	5.39
K. H.	42.88	37.50	17.77	14.72	25.11	22.78	3.66	3.04	6.61	6.05
W. H.	40.09	39.41	19.61	18.18	20.48	21.23	3.72	3.43	6.56	6.25
M. R.	34.70	30.04	14.30	14.13	20.40	15.91	3.51	2.95	6.03	5.47
F. N.	39.41	35.94	17.10	14.13	22.31	21.81	3.89	3.32	6.95	6.63
Mean	38.18	35.22	17.33	14.96	20.85	20.25	3.50	2.99	6.17	5.80
S. D.	3.01	3.29	1.70	1.45	2.84	2.46	0.28	0.32	0.54	0.51
Hypertensives										
L. E.	33.12	30.51	13.33	13.02	19.79	17.49	2.81	2.92	5.11	5.30
J. J.	38.75	34.09	15.70	14.42	23.05	19.67	3.36	2.50	6.04	5.16
R. D.	32.75	30.79	15.65	13.47	17.10	17.32	2.73	2.66	5.51	5.59
R. M.	38.11	35.77	17.45	14.19	20.66	21.58	3.61	2.84	6.16	4.99
C. S.	42.27	38.75	18.97	18.56	23.30	20.19	3.43	3.34	6.44	6.51
C. S.	36.90	32.77	14.73	12.93	22.17	19.84	2.53	2.64	5.19	5.53
M. M.	42.27	40.09	18.56	16.62	23.71	23.47	3.21	3.26	7.28	7.00
C. McM.	33.94	33.12	15.62	15.37	18.32	17.77	3.22	3.38	5.76	6.04
Mean	37.26	34.49	16.25	14.82	21.01	19.67	3.11	2.94	5.94	5.77
S. D.	3.82	3.50	1.93	1.95	2.45	2.15	0.38	0.34	0.71	0.70

of the technique is high, it provides a convenient and relatively reliable means of following change in the extracellular space. Our own data were insufficient to permit adequate testing of the antipyrine technique by this method, but preliminary examination of other data from this laboratory[10] indicates that its reliability is somewhat greater than that of the thiocyanate method.

RESULTS

The fluid spaces were approximately the same in the normotensive and hypertensive subjects before the sweating procedure was carried out. The small difference between the mean values for the two groups (Table I) is attributable to the small difference in their mean body weights. Determination of the plasma volume, blood volume, and thiocyanate space was carried out in the larger group of hypertensive subjects (23 in all), the results being approximately the same as in the smaller group.

The most significant result of the experiments is a negative one: there was no appreciable change in blood pressure after the sweating procedure, although there was a very considerable loss of body water (Table II). This was true for the hypertensive as well as for the normotensive subjects. During the sweating procedure, the blood pressure usually declined in both groups as the body temperature rose. Within an hour after the sweating procedure was terminated, however, the blood pressure invariably returned to the resting level, although the subjects were not permitted to eat or drink during this period.

The loss of weight during the sweating procedure was attributable almost entirely to loss of body water, mostly from the thiocyanate space. The mean

TABLE II. DIFFERENCES IN BODY WEIGHT, FLUID COMPARTMENTS, AND BLOOD PRESSURE INDUCED BY SWEATING PROCEDURE

SUBJECTS	BODY WT. (KG.)			BLOOD PRESSURE (MM. HG)		DECR. BODY WATER (LITERS)	DECR. SCN SPACE (LITERS)	DECR. ICF (LITERS)	DECR. BLOOD VOL. (LITERS)
	BEFORE	AFTER	DIFF.	BEFORE	AFTER				
Normotensives									
E. S.	74.50	69.99	4.51	134/54	116/70	3.85	1.74	2.11	0.30
J. L.	78.85	74.81	4.04	122/50	–	2.84	4.09	+1.25	0.22
J. A.	65.70	62.06	3.64	116/70	–	–	3.17	–	0.31
K. H.	85.80	80.65	5.15	120/70	114/70	5.38	3.05	2.33	0.56
W. H.	77.00	73.40	3.60	116/70	110/64	0.68	1.43	+0.75	0.31
M. R.	64.05	60.75	3.30	134/76	128/70	4.66	0.17	4.49	0.56
F. N.	64.60	61.20	3.40	124/70	104/70	3.47	2.97	0.50	0.32
Mean	72.93	68.98	3.95	124/66	114/69	3.48	2.37	1.24	0.37
S. D.	8.36	7.82	0.67			1.63	1.33	2.15	0.14
Hypertensives									
L. E.	58.50	56.40	2.10	190/140	225/145	2.61	0.31	2.30	+0.19
J. J.	74.30	70.55	3.75	160/105	138/100	4.66	1.28	3.38	0.88
R. D.	57.90	55.81	2.09	162/110	138/106	1.96	2.18	+0.22	+0.88
R. M.	68.40	65.90	2.50	174/110	180/94	2.34	3.26	+0.92	1.17
C. S.	86.00	83.00	3.00	158/95	152/76	3.52	0.41	3.11	+0.07
C. S.	76.80	74.53	2.27	148/98	138/89	4.13	1.80	2.33	+0.34
M. M.	75.90	73.40	2.50	153/110	150/110	2.18	1.94	0.24	0.58
C. McM.	66.00	63.63	2.37	225/150	248/158	0.80	0.25	0.55	+0.28
Mean	70.48	67.90	2.57	171/115	171/110	2.78	1.44	1.35	0.17
S. D.	9.64	9.33	0.56			1.26	1.07	1.63	0.56

decline in total body water (antipyrine space) closely approximated the mean decline in body weight for the two groups, but in some individual subjects there were wide discrepancies between the two values. The intracellular compartment (antipyrine space-thiocyanate space) apparently made some contribution to the fluid loss, but the amount was less than that contributed by the extracellular compartment (thiocyanate space). The amount of decrease in the blood volume was almost insignificant.

DISCUSSION

The possibility that loss of body water with relatively small loss of electrolytes may cause decline of the blood pressure in hypertensive patients thus receives no support from the foregoing experiments. Rise in body temperature, induced by a hot environment, causes some lowering of the blood pressure, but the effect is temporary.

The average total decrease in thiocyanate space, after six weeks on the rice-fruit diet, was, in our experience, 2.69 liters, but the depressor effect observed in four of the seven hypertensive subjects was apparent within a week or two, and well before the maximum decline in thiocyanate space was observed. In the present study, the average decrease in thiocyanate space induced by sweating was 1.44 liters in the hypertensive group, yet there was no significant decline in blood pressure (once the body temperature had returned to normal). This amount, although considerably less than that seen in the group receiving the rice-fruit diet, was certainly comparable to that seen in the early weeks of the dietary test period, at a time when the blood pressure had declined markedly in four of the hypertensive subjects.

The possibility that changes in blood volume are related to changes in blood pressure are not excluded by the present experiments. There is a slight suggestion in the data presented in Table II that such a relationship may exist, but the number of experiments is too small to prove or disprove the point.

The sweating procedure produced a rise in the concentration of serum electrolytes, but the relative concentrations did not change. The Na/K ratio, for example, was substantially the same before and after the sweating. Some loss of sodium chloride undoubtedly occurred but, as Adolph points out, sweat is such a dilute solution that ". . . its formation robs the body almost exclusively of water." The amount of salt loss in our subjects, although relatively small, does not appear to have been the same in the two groups of subjects. The data suggest, but do not prove, that the normotensives lost more than the hypertensives.

The situation with regard to the rice-fruit diet is quite different, as shown by Watkin and associates.[2] In spite of the very significant decrease in thiocyanate space, the concentrations of serum sodium and potassium remain substantially unchanged, while that of serum chloride declines. Considerable loss of electrolyte is, therefore, apparent. In terms of milliequivalents of sodium and chloride per liter of reduction in thiocyanate space, the loss of electrolytes was over twice as high in patients treated with the rice-fruit diet as in those subjected to sweating. Since the diet does exert a depressor effect, while the sweating procedure (of a degree producing significant decrease in thiocyanate space) does not, it seems likely that the rice-fruit diet directly depends, to some extent at least, on alterations in serum electrolytes for its effect.

When desoxycorticosterone is used to induce sodium retention in hypertensive patients, there is an immediate rise in blood pressure, as shown by Perera.[11] As the administration of the drug is continued, however, evidence of salt and water retention disappears and in some, but not in all, hypertensive patients the blood pressure returns to control levels. Perera interprets these findings to mean that some factor other than salt and water retention must be invoked to explain the effect of desoxycorticosterone on the blood pressure. It seems probable, on the basis of Perera's work and of our own, that several factors, including change in salt and water content, are involved, and that no single factor, acting alone, is capable of producing significant and enduring decrease in the blood pressure of hypertensive patients.

Although both the rice-fruit diet and the sweating procedure as used by us induce diminution in the thiocyanate space, they do it at very different rates. Days to weeks are required for the diet to cause its maximum reduction. The sweating procedure, on the other hand, caused substantial reduction in a few hours. It is possible that this time factor may in some way account for the difference in the effects on the bood pressure of the two techniques. This possibility, however, has not been investigated.

Finally, in our hands the rice-fruit diet caused definite loss of body tissue, while the sweating procedure did not. It seems unlikely, however, that the rice-fruit diet depends on loss of body tissue for its depressor effect. Others have shown that the diet may exert a depressor effect even when there is no, or

minimal, loss of body tissue.[2] Also, weight reduction alone usually causes relatively insignificant fall in the blood pressure of severely hypertensive patients, while the rice-fruit diet not infrequently produces a dramatic decline.

CONCLUSIONS

1. The plasma and total blood volumes, the thiocyanate space, and the antipyrine space are normal in patients with essential hypertension.

2. Dehydration, unaccompanied by large loss of electrolytes, has no lasting depressor effect in hypertensive patients.

REFERENCES

1. Chapman, C. B., Gibbons, T. B., and Henschel, A.: The Effect of the Rice-Fruit Diet on the Composition of the Body, New England J. Med. **243**: 899, 1950.
2. Watkin, D. M., Froeb, H. F., Hatch, F. T., and Gutman, A. B.: Effects of Diet in Essential Hypertension. II. Results With Unmodified Kempner Rice Diet in Fifty Hospitalized Patients, Am. J. Med. **9**: 441, 1950.
3. Gibson, J. G., and Evans, W. A., Jr.: Clinical Studies of the Blood Volume. I. Clinical Application of a Method Employing the Azo Dye "Evans Blue" and the Spectrophotometer, J. Clin. Investigation **16**: 301, 1937.
4. Gibson, J. G., and Evelyn, K. A.: Clinical Studies of the Blood Volume. IV. Adaptation of the Method to the Photoelectric Calorimeter, J. Clin. Investigation **17**: 153, 1938.
5. Crandall, L. A., Jr., and Anderson, M. X.: Estimation of the State of Hydration of the Body by the Amount of Water Available for the Solution of Sodium Thiocyanate, Am. J. Digest. Dis. & Nutrition **1**: 126, 1934.
6. Soberman, R., Brodie, B. B., Levy, B. B., Axelrod, J. Hollander, V., and Steel, J. M.: The Use of Antipyrine in the Measurement of Total Body Water in Man, J. Biol. Chem. **179**: 31, 1949.
7. Gregersen, M. I.: Blood Volume, Ann. Rev. Physiol. **13**: 397, 1951.
8. Leavitt, M. F.,: and Gaudino, M.: Measurement of Body Water Compartments, Am. J. Med. **9**: 208, 1950.
9. Keys, A., Brozek, J., Henschel, A., Mickelsen, O., and Taylor, H. L.: The Biology of Human Starvation, Minneapolis, 1950, The University of Minnesota Press.
10. Anderson, J. T., Henschel, A., Taylor, H. L., and Keys, A.: In preparation.
11. Perera, G. A.: Effect of Continued Desoxycorticosterone Administration in Hypertensive Subjects, Proc. Soc. Exper. Biol. & Med. **68**: 48, 1948.

RESULTS IN THE HEMAGGLUTINATION TEST FOR TUBERCULOSIS

WILLIAM HENTEL, M.D.,* AND G. D. GUILBERT, M.D.
KERRVILLE, TEXAS

IN 1948, Middlebrook and Dubos[1] described the method, technique, and results of testing the products of tubercle bacilli against antibodies in human sera. This was interpreted as a new means of detecting antibodies in the serum of patients with tuberculous infections. In 1950, Smith and Scott[2, 3] published two papers describing modification of the original method as well as interpretation of their results.

Our procedure followed the modification of Scott and Smith,[2] with utilization of Lederle 4X Tuberculin. Over a period of ten months we studied a total of 475 separate individuals. Of this entire study, Group A (130 individuals) consisted of employees of the hospital who were presumed to be nontuberculous. This was substantiated by routine physical examinations and chest roentgenograms necessary for all federal employees. These were called "nontuberculous." The 345 patients with known pulmonary tuberculosis undergoing the hemagglutination test were broken down into four different categories. Group B (78 cases) consisted of patients who had undergone extensive treatment and were ready for discharge as arrested. These were called the "clinically inactive group." Group C (48 cases) consisted of recently admitted patients having roentgenogram findings suggestive of tuberculous infection, but in whom no positive sputum or gastric cultures had as yet been obtained. These were called the "questionable activity group." Group D (112 cases) consisted of patients with known pulmonary tuberculosis who had never received streptomycin or para-aminosalicylic acid. These were called "known active tuberculosis without antibiotics." Our last group, Group E (107 cases), was composed of patients with proved active pulmonary tuberculosis who had been vigorously treated with both streptomycin and para-aminosalicylic acid. These were called "Known active tuberculosis with antibiotic therapy."

Our presumed nontuberculous group of employees (130 individuals) was also investigated regarding the tuberculin reaction. Of this group, 128 individuals showed a positive tuberculin reaction, and two individuals were negtive.

All bloods obtained were listed by number only and the individual performing the hemagglutination procedure was uninformed of the patient's identity. A titer of 1:8 or less was considered within normal limits. Titers ranging from 1:16 or higher were considered to be indicative of tuberculous activity.

RESULTS

Group A (Nontuberculous) 130 Cases.—Of this group, 123 cases or 94.6 per cent of the individuals showed a titer of 1:8 or less. (Table I, Group A.) Seven individuals, or 5.4 per cent, showed a hemagglutination titer of 1:16 or above. If one were to consider this group alone, the results would seem to be most encouraging toward utilization of the hemagglutination test as a method of mass tuberculosis screening.

From the Veterans Administration Hospital, Legion Branch, Kerrville, Texas.

Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

Received for publication, Sept. 27, 1951.

*Now stationed at Veterans administration Hospital, Albuquerque, N. M.

TABLE I

GROUP	TITER	NEGATIVE HEMAGGLUTINATION GROUP					POSITIVE HEMAGGLUTINATION GROUP						
	NO. OF CASES	NEG.	1:2	1:4	1:8	PER CENT	1:16	1:32	1:64	1:128	1:256	1:512	PER CENT
A	130	62	30	15	16	94.6	5	2	0				5.4
B	78	26	13	15	19	93.6	2	2	1	0			6.4
C	48	14	10	15	4	89.6	2	2	1	0			10.4
D	112	18	8	17	18	54.5	12	19	13	7	0		45.5
E	107	12	15	25	25	71.9	8	11	7	2	2	0	28.1
D & E combined	219	30	23	42	43	63.0	20	30	20	9	2	0	37.0

Group B (Clinically Inactive) 78 Cases.—The group on the whole demonstrated results consistent with that found by Middlebrook and Dubos,[1] as well as Smith and Scott.[3] Seventy-three cases or 93.6 per cent showed a titer of 1:8 or less, whereas five cases or 6.4 per cent showed a titer of 1.16 or higher (Table I, Group B). The results in this group would indicate a decrease in the titer of the hemaglutination test for individuals whose tuberculous disease is arrested, or at least inactive.

Group C (Questionable Activity) 48 Cases.—Although roentgenogram findings suggestive of pulmonary tuberculosis were present, none of this group were proved to have tuberculosis by positive sputum or gastric cultures. The majority of individuals (37 patients) were considered in the class with minimal involvement, but eleven patients were considered to have chronic fibrotic pulmonary tuberculosis. Forty-three cases or 89.6 per cent showed a hemagglutination titer of 1:8 or less. Five cases, or 10.4 per cent, showed a titer of 1:16 or higher (Table I, Group C). Although this group is difficult to evaluate, one must consider a 10 per cent false-positive reaction as being too high for adequate screening purposes.

Group D (Known Active Pulmonary Tuberculosis Without Antibiotics) 112 Cases.—All patients were proved tuberculosis by roentgenogram and culture studies. This group of patients was considered hopeless, and most of the patients were in the terminal stages of the disease. Sixty-one cases, or 54.5 per cent, showed a hemagglutination titer of 1:8 or less, whereas 51 cases, or 45.5 per cent, showed a titer of 1:16 or higher (Table I, Group D). This group demonstrates poor reaction to the hemagglutination test. However, Smith and Scott[2] state that negative results are frequently found in patients with far-advanced disease.

Group E (Known Active Pulmonary Tuberculosis With Antibotic Therapy) 107 Cases.—These patients were all treated with a combination of streptomycin or dihydrostreptomycin and para-aminosalicylic acid. Dosage was governed by the protocols as established by the Eighth Veterans Administration, Army and Navy Conference on the Chemotherapy of Tuberculosis.[4] The dosage under these protocols was either 1 Gm. streptomycin (SM) or dihydrostreptomycin (DHSM) and 12 Gm. para-aminosalicylic (PAS) acid daily for one hundred and twenty days, or 1 Gm. SM twice a week and 12 Gm. PAS daily

for one hundred and twenty days, the particular regimen being randomized according to the hospital registration number. Seventy-seven cases, or 71.9 per cent, showed a titer of 1:8 or less. Thirty cases, or 28.1 per cent, showed a titer of 1:16 or higher (Table I, Group E).

Considering the statement that far-advanced cases without antibiotic therapy often show negative hemagglutination results, we can only postulate that the role of antibiotics in treatment of pulmonary tuberculosis may be manifested by the reduction in titer. As seen in Table I, Group D, 61 patients, or 54.5 per cent, of the group that *did not* have antibiotics had negative hemagglutination results (1:8 or lower), while of those who *did* have antibiotics (Table I, Group E), 77 patients, or 71.9 per cent, had negative hemagglutination results. Thus there were 17.4 per cent more patients who had negative agglutination results in the antibiotic-treated group than in the nonantibiotic-treated group. The actual effect of antibiotic therapy on the hemagglutination titer is not known and cannot be evaluated from this small series of patients.

Combining both groups D and E for an over-all hemagglutination test in known active pulmonary tuberculosis, treated with and without antibiotics, the greater percentage is found to be less than 1:8 titer. This combined group had a total of 219 cases. One hundred and thirty-eight cases, or 63 per cent, showed a hemagglutination titer of 1:8 or less, whereas eighty-one cases, or 37 per cent, demonstrated a titer of 1:16 or higher. In other words, more patients in the definitely active group had a negative agglutination titer than had positive titers (Table I, D and E).

SUMMARY

The present status of this test is that it is not of sufficient accuracy to determine the activity of pulmonary tuberculosis.

We are indebted to Mr. James R. Copeland for technical assistance in performance of the hemagglutination test.

REFERENCES

1. Middlebrook, G., and Dubos, R. J.: Specific Serum Agglutination of Erythrocytes Sensitized with Extracts of Tubercle Bacilli, J. Exper. Med. **88**: 521-528, 1948.
2. Scott, N. B., and Smith, D. T.: A Simple Modification of the Middlebrook and Dubos Hemagglutination Test for Serum Antibodies to Products of Tubercle Bacilli, J. LAB. AND CLIN. MED. **35**: 303-307, 1950.
3. Smith, D. T., and Scott, N. B.: Clinical Interpretation of the Middlebrook-Dubos Hemagglutination Test, Am. Rev. Tubere. **62**: 121, 1950.
4. Minutes of the Eighth Veterans Administration, Army and Navy Streptomycin Conference, Nov. 10 to 13, 1949.

THE EARLY DEVELOPMENT OF ANATOMIC AND BLOOD CHEMISTRY CHANGES IN THE NEPHROTIC SYNDROME IN RATS

WALTER HEYMANN, M.D., AND DONALD B. HACKEL, M.D.
CLEVELAND, OHIO

THE chronic renal disease induced in rats by the intravenous injection of nephrotoxic serum obtained from rabbits simulates the nephrotic syndrome as it is observed in infants and children.[1] The pathogenesis of the human disorder is poorly understood. This is possibly due to the insidious onset of the disease in children. It is likely that the nephrosis has existed several weeks before the first symptoms—edema and ascites—are noted. Consequently, the early changes in the kidneys and in the chemical composition of blood thus escape detection. This communication describes the histologic lesion in the kidneys and the variation in the concentration of the blood urea nitrogen, and creatinine, and serum protein, cholesterol, lecithin, and total lipids as they were noted during the first hours and days after the intravenous injection of anti-rat-kidney serum obtained from rabbits.

METHODS

The experimental procedures used have been previously described.[1, 2] Rats of the Long-Evans strain were used. The nephrotoxicity of anti-kidney sera was tested in preliminary experiments by biologic assay. The amount of serum needed to produce a severe disease thus was established and standardized according to estimated kidney weight.[1] In contradistinction to former practice, the dosage was administered in one single intravenous injection. The animals were then sacrificed by decapitation at intervals from thirty minutes to ninety-six hours. Urine was collected for quantitative protein determination. Serum from normal rabbits was used in place of the nephrotoxic serum as a control.

RESULTS

Early Anatomic Changes.—Kidneys of forty-six rats injected with nephrotoxic serum and kidneys from nine control rats that had been injected with normal rabbit serum were examined. Formalin-fixed sections were stained with hematoxylin and eosin. Basement membrane changes were demonstrated by the Lee-Brown[3] or periodic acid-Schiff[4] techniques.

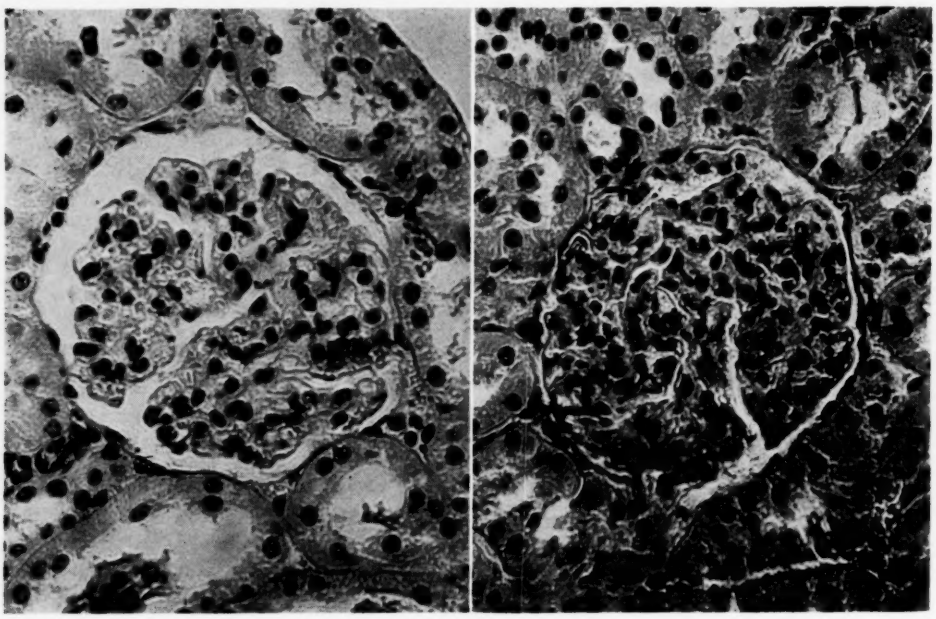
Histologic changes were present in the kidneys of forty-five of the forty-six rats injected with nephrotoxic serum. Only one rat was found to have microscopically normal kidneys. The earliest histologic change was found to be a marked accumulation of polymorphonuclear leukocytes within the glomeru-

From the Departments of Pediatrics and Pathology, Western Reserve University School of Medicine, and the Department of Pathology, Cleveland City Hospital.

*Aided by grants of the Life Insurance Medical Research Fund and the Louis D. Beaumont Trust.

Received for publication, Oct. 1, 1951.

lar tufts (Figs. 1, *A* and 1, *B*). This was observed as early as one-half hour after injection of the nephrotoxic serum, was most marked within one to two hours, and had largely disappeared after eight hours. The reaction was seen in the one rat killed after one-half hour, in both of two rats killed after one hour, in one rat killed after three hours, in six of seven rats killed after four hours, in four of six rats killed after six hours, in four of five rats killed after eight hours, and was absent in all rats killed thereafter. After one to two hours the capillary basement membrane in all kidneys was thickened, and there was swelling of the endothelial cells. This resulted in obliteration of the capillary lumens and an apparent decrease in vascularity of the glomerular tufts.



A. *B.*

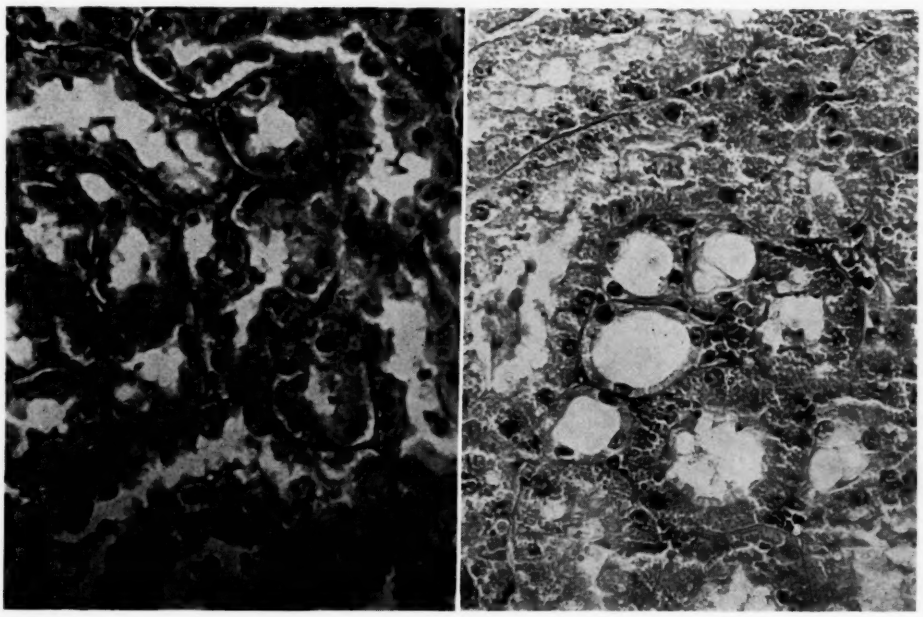
Fig. 1.—Glomerulus of a normal rat (*A*) contrasted with that of a rat killed one hour after injection of nephrotoxic serum (*B*). Note the clear capsular space, the fine capillary loops and small number of nuclei in (*A*). The obliteration of the capsular space and of the capillary loops in (*B*) is due to the presence of a large number of polymorphonuclear leukocytes, to the thickening of the capillary basement membrane and swelling of the endothelial cells. (Hematoxylin and eosin, ×612.)

The first tubular changes were noted after four hours. In hematoxylin and eosin stained sections, many eosinophilic colloid casts were observed within the tubular lumens. In numerous instances the epithelial cells of the convoluted tubules presented the picture of hyaline droplet "degeneration" (Figs. 2, *A* and 2, *B*), with coarse granules having the same staining characteristics as the colloid casts in the lumens.

Frozen sections of kidneys were stained with Sudan IV. No fat was present until eighteen hours after injection of nephrotoxic serum. It was present in the renal tubules of eleven of the twenty-two rats examined from this time through

ninety-six hours. The proximal convoluted tubules were most markedly involved, but the distal convoluted and collecting tubules were also frequently affected, as were the glomeruli. The amount of fat in the tubular epithelium could not be directly correlated with the degree of hyperlipemia, but in only one instance was fat present in the absence of definite hyperlipemia.

In all but two rats the interstitial tissue was normal. In one rat killed after four hours and with one rat killed after six hours there were small focal perivascular collections of lymphocytes and plasmocytes.



A. *B.*

Fig. 2.—Renal tubules of a normal rat (*A*) contrasted with a kidney of a rat killed eighteen hours after injection of nephrotoxic serum (*B*). The latter shows marked colloid droplet "degeneration" of the tubules. (Hematoxylin and eosin, ×612.)

The kidneys of nine control rats that were injected with normal rabbit serum showed no abnormal changes.

Early Blood Chemistry Changes.—The values for blood urea nitrogen and creatinine, serum protein, cholesterol, phospholipids, and total lipids were obtained at various intervals after injection of nephrotoxic serum in sixty-six animals. They have been plotted as average values in Fig. 3.

The BUN in normal rats varies between 15 and 23 mg. per cent. It rises slightly within the first twenty to thirty minutes. From two to four hours after the injection of nephrotoxic serum, the values increase reaching a peak after twenty-four to forty-eight hours. They then slowly decrease and frequently reach normal levels within the next two to three weeks, in spite of continued massive proteinuria. The systolic blood pressure usually remains normal

through this period. Occasionally it may be slightly elevated, but hardly ever above 140 mm. Hg. The blood creatinine concentration remains within normal range (0.8 to 1.8 mg. per cent) throughout the initial phase of the disease. Changes during the later stages of the disease have been described previously.[1, 2] The total lipids rise slowly within the first two to four hours, and continue to increase well above normal range (180 to 400 mg. per cent) after four hours. The changes in serum cholesterol concentration are similar. Its normal range is 50 to 100 mg. per cent. From six to eight hours after the injection of heteronephrotoxins, hypercholesterolemia is present and increases progressively during the next days and weeks. The phospholipids also increase above normal range (100 to 150 mg. per cent) beginning six to eight hours after injection, and the curve is similar to the one obtained for cholesterol and total lipids.

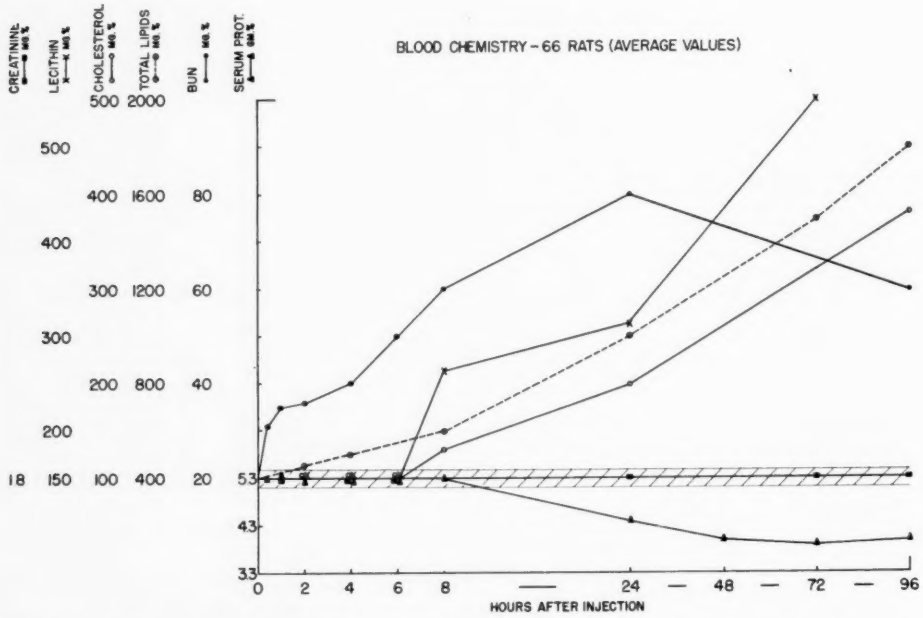


Fig. 3.—Blood chemistry changes during the first hours and days after the injection of nephrotoxic sera in rats. All values outside of the lined area are beyond the normal range.

After the first four to eight weeks the hyperlipemia may decrease, but the values stay above normal range as long as proteinuria is present. The average values for the serum protein concentration decrease below normal (5.2 to 5.9 Gm. per cent) eight to twenty-four hours after the injection of nephrotoxic sera, and an occasional low value is noted as early as four hours. Between four and eight hours ten values were between 4.6 and 5.3 Gm. per cent, while fifteen were between 5.3 and 6.8 Gm. per cent. During the same time interval only one of nineteen total lipid values, four of eleven phospholipid, and eight of seventeen cholesterol values remained within normal range. The serum protein values

decrease during the first three days and usually increase during the further course of the disease in accordance with the severity of the proteinuria.

Fig. 4 shows the correlation of serum protein values to the excretion of urinary proteins within the first hours and days after renal disease has been induced. It can be seen that the serum protein concentration decreases as the excretion of proteins in the urine increases. In ten instances, indicated by dots in Fig. 4, the hypoproteinemia is so marked that it is unlikely that it can be explained by the relatively small amount of protein found in the urine.

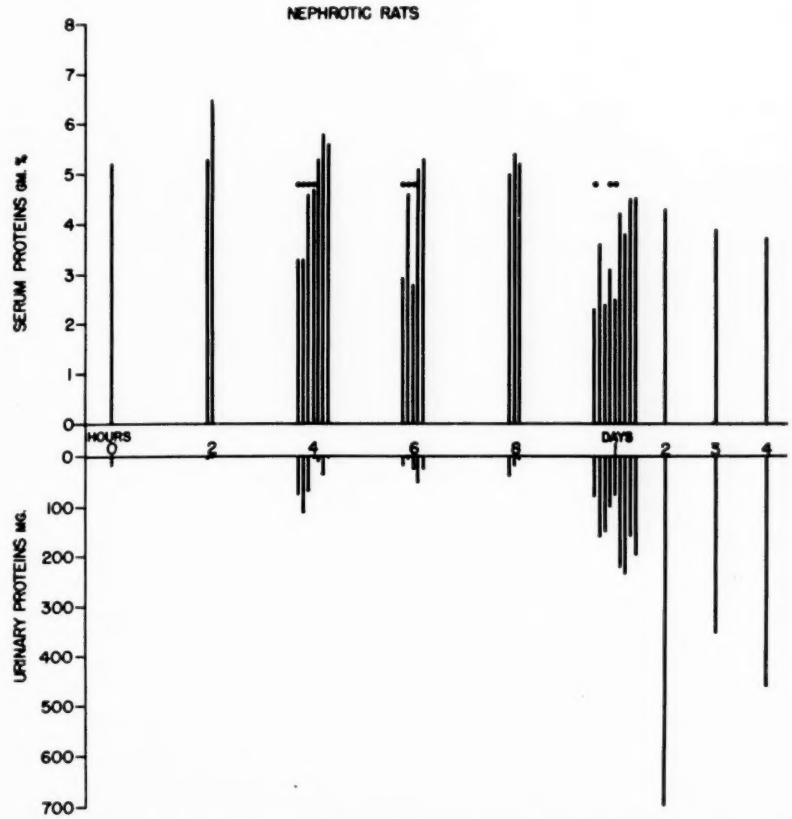


Fig. 4.—Correlation of serum protein concentration and degree of proteinuria during the first hours and days after the injection of nephrotoxic sera in twenty-nine rats. Each bar represents the values for one rat. The values at "0" hours are the normal concentrations. The serum protein concentration of control rats varied between 5.2 and 5.9 Gm. per 100 ml. The urinary protein excretion is given in milligrams over the period covered.

In order to ascertain the effect of serum per se on the BUN, eight animals were injected with corresponding amounts of sera obtained from untreated rabbits. An increase in the blood urea nitrogen concentration to maximum values of 30 to 32 mg. per cent was observed after two hours, and after four to six hours the values had returned to the normal range. Blood creatinine concentration, serum cholesterol, lecithin, and total lipid values did not show any change.

DISCUSSION

After the injection of inactive rabbit serum no histologic abnormalities were noted in the kidneys. This suggests that the changes that occur after nephrotoxic sera are used are due to the action of the renal antibodies. It is of considerable interest that the glomeruli were affected first, while the tubular changes followed four to six hours thereafter. Based on extensive studies of renal function in the nephrotic syndrome of children, Metcoff, Kelsey, and Janeway[5] arrived at a similar view: that this disorder affects the entire nephron even though the glomerulus is a primary site of the disease process. In our experience the extent of the initial histologic glomerular changes does not reflect the intensity of the disease at onset, nor does it necessarily imply a progression of the glomerular injury to fibrosis or atrophy. This has been shown in a previous communication.[2] It has been shown that the initial antigen antibody reaction takes place in the glomeruli.[6-9] It may be assumed that the subsequent injury need not always manifest itself initially by visible anatomic alterations, since in a few rats with severe disease associated with anasarca, massive proteinuria, hypoproteinemia, and hyperlipemia, the glomeruli appeared normal.[1] One may be inclined to apply this knowledge to the so-called "pure" form of lipemic nephrosis as it is observed in children and in the experimental disease of rats.

An abnormal amount of fat in the tubular cells was not seen until eighteen hours after the injection of nephrotoxic serum. In only one case was fat seen in the tubular cells without hyperlipemia. However, there were numerous instances in these early stages in which hyperlipemia was present, yet no fat could be demonstrated in the tubules. As hyperlipemia per se does not necessarily lead to deposition of fat in the renal tubules,[11] the addition of some injury to these cells has to be presumed to explain the deposition of fatty substances in the tubular epithelium.

The azotemia of the initial phase of the nephrotoxic disease in rats deserves some comment. The intravenous injection of both normal and nephrotoxic rabbit serum increases the BUN values within the first two hours to similar degrees. It is therefore likely that during the first two hours the azotemia is due to the parenteral administration of foreign protein rather than to the effect of the renal antibodies. The progressive and marked increase of blood urea nitrogen that is observed after two hours is, however, probably due to the renal lesion. Complete renal ischemia was established in six rats by occluding the renal artery and vein of both kidneys with bulldog clamps.[10] The BUN rose at first in these animals at the same rate as in those injected with nephrotoxic sera. After seven to eight hours, the azotemia of nephrotic rats no longer increased as rapidly as in animals in whom complete cessation of renal blood flow had been established.

The functional impairment of the glomeruli in the nephrotic rats may be severe for a matter of hours to a few days. However, it must also be reversible,

as a decrease of the azotemia to normal levels during the following weeks and months is the rule. In contradistinction to this observation is the persistence and irreversibility of the azotemia in late stages of the disease. In children with the nephrotic syndrome, we also have occasionally observed an initial, reversible azotemia. The importance of this observation in the prognostic evaluation is obvious, and a survey of suitable cases is being undertaken.

The observation that the hyperlipemia develops prior to or possibly together with—but certainly not after—the hypoproteinemia provides more[12] convincing evidence against the assumption that the hypoproteinemia is the cause for the nephrotic hyperlipemia.[13]

The further observation that the amounts of protein recovered from the urine of nephrotic rats cannot always explain the degrees of hypoproteinemia noted during the first few hours of the disease has to be evaluated with caution. Changes in plasma volume and the unknown amount of proteins lost in the lumen of renal tubules might account for this discrepancy.

SUMMARY

Anti-rat-kidney serum obtained from rabbits was given by intravenous injection to sixty-six rats. The histologic changes in the kidneys and the changes in blood chemistry values were studied during the first hours and days after the injection.

The glomeruli may show histologic changes as early as one-half hour after the injection. The tubules show no abnormalities until four hours. After this interval, hyaline casts are often seen and hyaline droplet "degeneration" of the tubular cells is frequently marked. Fat in the tubular cells is not seen until eighteen hours.

The BUN may start to rise as early as one-half hour after the injection of nephrotoxic serum. This is probably due to the intravenous injection of foreign protein material. Renal azotemia develops after two to four hours. Total lipids begin to increase after two to four hours, serum cholesterol and phospholipids after six to eight hours. The serum protein concentration begins to decrease after eight to twenty-four hours.

No histologic or comparable chemical abnormalities are seen in nine control rats injected with normal rabbit serum and killed at similar intervals.

REFERENCES

1. Heymann, W., and Lund, H. Z.: The Nephrotic Syndrome in Rats, Pediatrics **7**: 691, 1951.
2. Heymann, W., Lund, H. Z., and Hackel, D. B.: The Nephrotic Syndrome in Rats with Special Reference to the Progression of the Glomerular Lesion and to the Use of Nephrotoxic Sera Obtained from Ducks, J. LAB. & CLIN. MED. Feb. issue.
3. Lee-Brown, R. K., and Laidley, J. W. S.: Some Observations on the Microscopic Anatomy of the Kidney, J. Urol. **21**: 259, 1929.
4. McManus, I. F. A.: The Periodic Acid Routine Applied to the Kidney, Am. J. Path. **24**: 643, 1948.
5. Metcoff, J., Kelsey, W. M., and Janeway, C. A.: The Nephrotic Syndrome in Children. An Interpretation of Its Clinical, Biochemical and Renal Hemodynamic Features as Varions of a Single Type of Nephron Disease, J. Clin. Investigation **30**: 471, 1951.

6. Pressman, D., Hill, R. F., and Foote, F. W.: The Zone of Localization of Anti-Mouse Kidney Serum as Determined by Radio Autographs, Science **109**: 65, 1949.
7. Solomon, D. H., Jardella, J. W., Fanger, H., Dethier, F. M., and Ferrebee, J. W.: Nephrotoxic Nephritis in Rats, J. Exper. Med. **90**: 267, 1949.
8. Heymann, W., Gilkey, C., and Salehar, M.: The Antigenic Property of Renal Cortex, Proc. Soc. Exper. Biol. & Med. **73**: 385, 1950.
9. Krakower, C. A., and Greenspan, S. A.: Localization of the Nephrotoxic Antigen within the Isolated Renal Glomerulus, Arch. Path. **51**: 629, 1951.
10. Koletsky, S., and Gustapon, G. E.: The Effects of Temporary Cessation of Renal Blood Flow in Rats, J. Clin. Investigation **26**: 1072, 1947.
11. Chapman, F. D., and Kinney, T. D.: Hyperlipemia, Am. J. Dis. Child. **62**: 1018, 1941.
12. Heymann, W., and Startzman, V.: Lipemic Nephrosis, J. Pediat. **28**: 117, 1946.
13. Fishberg, E. H., and Fishberg, H. M.: The Mechanism of the Lipemia of Bleeding, Proc. Soc. Exper. Biol. & Med. **25**: 296, 1928.

STUDIES ON THE TECHNIQUE OF TRANSPORTING THROAT SWABS TO THE LABORATORY FOR THE ISOLATION OF PNEUMOCOCCI*

DAVID A. STEVENS, A.B.
CLEVELAND, OHIO

STUDIES on the isolation of pneumococci from throat swabs were undertaken because carrier rates for these organisms from individuals comprising a population of normal families[1] were lower than the results of similar studies on normal soldiers.[2] The technique employed had not differed from the method used previously,[3] except that the swabs were obtained in the home and transported to the laboratory in dry test tubes rather than in tubes containing broth.

Data have been obtained which show that pneumococci often may not be detected on throat swabs transported to the laboratory in dry test tubes, that the losses may be appreciable, but that viability of pneumococci may be maintained if the swabs are transported in broth.

METHODS

Two sterile dry cotton swabs were passed firmly across the tonsillar areas and the posterior pharyngeal wall of each person cultured. After variable intervals of time, each of the swabs was placed in 5 ml. of tryptose phosphate broth containing 0.1 ml. of defibrinated horse blood and the broth aspirated twenty times with a 5 ml. pipette. The culture was incubated at 37° C. for four hours with the swab in place, after which 0.5 ml. of the culture was inoculated intraperitoneally into one or more Swiss mice (eight to ten weeks old). Peritoneal washings from mice dying within forty-eight hours were examined for pneumococci by the Neufeld Quellung method employing antisera for types 1 to 33.* Mice surviving forty-eight hours were sacrificed, and the peritoneal washings cultured on a horse blood agar plate. Neufeld typing was attempted employing the resultant growth on the plate or a subculture in horse blood broth, or both.

In two of the three experiments each culture was inoculated into three mice. The first mouse inoculated was arbitrarily selected to represent results obtained by inoculating one animal; the first and second mouse inoculated represented the collective results with two animals, etc. In the third experiment, each culture was inoculated into one mouse.

For purposes of analysis a given culture was scored for as many different pneumococcal types as were isolated—for example, Table III includes two specific types isolated from each of three of the ninety-one cultures obtained, hence there was a net total of nintey-four cultures.

From the Department of Preventive Medicine, School of Medicine, Western Reserve University.

*This investigation was supported in part through the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, Office of The Surgeon General, U. S. Army, and by grants from the Brush Foundation, the Cleveland Foundation, the S. P. Fenn Trust, and Mr. Philip R. Mather.

Received for publication, Oct. 1, 1951.

*Specific antisera were supplied by Lederle Laboratories and the New York State Department of Health, Division of Laboratories and Research.

RESULTS

Isolation of pneumococci from throat swabs stored in dry test tubes compared with immediate incubation of swab in blood broth.—Duplicate throat swabs were obtained from forty-two well persons by swabbing the throat with two swabs simultaneously. One swab was placed in blood broth and incubated immediately; the other was stored in a dry test tube at 21°C. for two and one-half hours before being placed in blood broth and incubated. After incubation of the cultures for four hours, each was inoculated into three mice.

The results (Table I) showed that twice as many strains of pneumococci were isolated from the swabs incubated immediately in blood broth as from swabs stored two and one-half hours in the dry state before incubation in blood broth. Examination of a second and third mouse increased the number of types of pneumococci isolated from both types of swabs.

TABLE I. COMPARISON OF DRY SWABS AND BROTH SWABS FROM 42 INDIVIDUALS FOR THE ISOLATION OF PNEUMOCOCCI.

NUMBER OF MICE EXAMINED*	NUMBER OF PNEUMOCOCCI ISOLATED	
	DRY SWAB	BROTH SWAB
1	4	10
2	5	11
3	6	12

*See text.

Storage of swab in broth compared with immediate incubation in blood broth.—Each of forty-five duplicate throat swabs was placed in 5 ml. of broth immediately. To one of each pair, 0.1 ml. of horse blood was added and the culture incubated at once; the other of each pair was stored at 21°C. for two and one-half hours when horse blood was added and the culture incubated. Each culture, after four hours of incubation, was inoculated into three mice. The results (Table II) showed that approximately the same number of types of pneumococci were isolated by each method.

Dry swab compared with swab in broth under field conditions.—Duplicate throat swabs were collected from ninety-one individuals in the home. One swab was placed in broth and the duplicate was placed in a dry tube. The delay in transporting the swabs to the laboratory varied from twenty minutes to two hours, and the temperature varied from approximately 19° C. to 25° C. Upon arrival at the laboratory, blood was added to the broth swab and blood broth to the dry swab. After four hours' incubation each culture was inoculated into a mouse.

Twenty-nine specific types of pneumococci were isolated from the ninety-one pairs of swabs; of these twenty-nine types, thirteen were isolated from both the dry and broth swabs. The organism was detected on broth swabs alone in thirteen instances and on dry swabs alone in three instances. Thus, 90 per cent (twenty-six of twenty-nine) of the total types of pneumococci isolated were detected from the swabs transported in broth, whereas only 55 per cent (sixteen of twenty-nine) were detected from the swabs transported in the dry state.

TABLE II. ISOLATION OF PNEUMOCOCCI FROM 45 DUPLICATE THROAT SWABS STORED IN BROTH AND INCUBATED EITHER IMMEDIATELY OR AFTER 2½ HOURS (DELAYED)

NUMBER OF MICE INOCULATED BY EACH METHOD*	NUMBER OF PNEUMOCOCCI ISOLATED BY METHOD OF INCUBATION		
	IMMEDIATE	DELAYED	BOTH†
1	12	14	17
2	13	15	18
3	15	16	19

*See text.
†Total number of different isolations from the two sets of cultures.

There were insufficient data to evaluate the possibility that certain types of pneumococci were more or less resistant to loss than other types under conditions of the experiments.

SUMMARY

The results of this study show that many pneumococci were not recovered from throat swabs when the swabs were either stored or transported to the laboratory in dry test tubes. Transporting or storing throat swabs in broth reduced such losses. Inoculation and examination of a second and third mouse increased the likelihood that pneumococci would be detected from a throat swab.

The technical assistance of Bernice Miraldi and Mary K. Dixon is gratefully acknowledged.

REFERENCES

1. Dingle, J. H., Badger, G. F., Feller, A. E., Hodges, R. G., Jordan, W. S. Jr., and Rammelkamp, C. H. Jr.: A Study of Respiratory Infections in Families, Trans. Assn. of Am. Phys. **62**: 99, 1949.
2. Commission on Acute Respiratory Diseases: Bacteriological Findings in Undifferentiated and Other Acute Respiratory Diseases, Medicine **26**: 465, 1947.
3. Robinson, R. L.: Studies on the Technique of Isolating Pneumococci from Throat Cultures by Mouse Inoculation, Am. J. Hygiene **50**: 343, 1949.

COLD HEMAGGLUTININS: EFFECT OF STORAGE AT –20° C.

A. E. Feller, M.D.*
Cleveland, Ohio

IT HAS been demonstrated repeatedly[1-8] that cold hemagglutinins in sera from patients with primary atypical pneumonia may decrease in titer or disappear after storage at ordinary refrigerator temperature (approximately 4° C.). In one report[7] it was noted that freezing and drying two sera resulted in the destruction or inactivation of cold hemagglutinins which had been present in exceptionally high titer. In another report[4] it was stated that a serum stored at –70° C. in sealed glass ampules maintained its titer for six months.

Data have been obtained which indicate that sera from patients with primary atypical pneumonia can be stored in the frozen state (–20° C.) for at least three years without loss in titer of cold hemagglutinins.

METHODS

Technique of Tests for Cold Hemagglutinins.—The technique employed has been presented in detail elsewhere.[9, 10] Titers of sera are expressed as the reciprocal of the highest final dilution of serum in the tube in which definite agglutination was detected. The original titer of a serum was that obtained in the first test performed within one month of the date the blood was drawn and served as the basis of comparison with subsequent titers obtained after storage at –20° C. The duration of storage at –20° C. was taken as the elapsed time in months from the date the blood was drawn to the date of test.

Sera.—Sera were removed from clots after twenty-four to forty-eight hours storage at room temperature, placed in glass vials with rubber stoppers, and stored at –20° C. Rapid prefreezing was not employed. For the tests, the sera were thawed in a 37° C. water bath, the desired quantity of serum removed, and the vials returned to –20° C. In most instances the vials were returned to –20° C. within a few hours, but occasionally certain of the vials were stored at 4° C. for a few days. With two exceptions (Table II), all sera were thawed and refrozen on one or more occasions for tests other than for cold hemagglutinins.

RESULTS

The influence of storage of sera at –20° C. on cold hemagglutinins was determined from the results of second tests performed on eighty-six single sera after varying periods of storage at –20° C. (Table I) and from the results of repeated tests on samples of two sera (Table II). Samples of the latter sera (Table II) were left undisturbed for the indicated period of storage and the tests were performed with erythrocytes from a single donor; the former sera (Table I) had been used for other purposes, and several donors of erythrocytes were employed.

From the Departments of Preventive Medicine and of Medicine and the University Hospitals, School of Medicine, Western Reserve University.

This investigation was supported in part through the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, Office of The Surgeon General, U. S. Army, and by grants from the Brush Foundation, the Cleveland Foundation, the S. P. Fenn Trust, and Mr. Philip R. Mather.

Received for publication, Oct. 17, 1951.

*Present address: University of Virginia, Department of Medicine, School of Microbiology, Charlottesville, Va.

TABLE I. NUMBER OF SERA SHOWING INDICATED CHANGE IN TITER OF COLD HEMAGGLUTININS AFTER VARIOUS PERIODS OF STORAGE

FOLD CHANGE IN TITER AFTER STORAGE*	MONTHS OF STORAGE					TOTAL NUMBER OF SERA
	1-6	7-12	13-18	19-24	31-36	
>+16					1†	1
+16	2	1			2	5
+8	2	2				4
+4	1	5	2	2	1	11
+2	3	6	2	1	5	17
0	6	7	3		2	18
−2	6	2	3		2	13
−4	2	8	1		1	12
−8	2	1		1	1	5
Total	24	32	11	4	15	86

*Original titer (see text) employed as baseline; 70 per cent of the sera had an original titer of 64 or higher.

†This serum was ± from the dilution of 1:4 through the dilution of 1:256 (titer < 4) on the original test and had a titer of 512 when tested thirty-one months later.

TABLE II. TITERS OF COLD HEMAGGLUTININS IN SAMPLES OF TWO SERA AFTER STORAGE AT 4° C. AND −20° C.*

DATE OF TEST	SERUM A (OBTAINED 3/24/47)		SERUM B (OBTAINED 4/16/47)	
	TEMP. OF STORAGE:		TEMP. OF STORAGE:	
	4° C.	−20° C.	4° C.	−20° C.
3/26/47	2048†			
4/17/47	2048	1024	512	
7/ 2/47	1024	2048	512	1024
7/24/47	512	1024	512	1024
8/27/47	512	512	256	512
11/ 4/47	512	2048	256	1024
2/24/48	256	1024	128	512
10/30/48	256	1024	64	256
4/ 9/49	256	1024	256	1024

*For storage each fresh serum was distributed to vials in volumes (0.6 ml.) sufficient for one test only. Thus, the date of test indicates the termination of a continuous and undisturbed period of storage of a single vial of serum at the indicated temperature. A single donor of the group O erythrocytes was employed for all tests and in each instance the erythrocytes were obtained on the day the tests were assembled.

†Titer of cold hemagglutinins.

The data show (Tables I and II) that, within the limits of variation commonly inherent in serologic tests involving agglutination of erythrocytes, there was no change in titer of cold hemagglutinins in sera stored at −20° C. for three years. It is believed that the changes in titer which were observed after storage were due to factors other than an actual change in the hemagglutinin content of the sera because increases in titer were observed approximately as frequently as decreases in titer (Table I). With the two sera in which technical variations were probably minimal (Table II), there was no consistent change in titer after two years of storage at −20° C. Data on these same two sera stored for two years at 4° C. suggest that there was a loss of titer in serum A and probably in serum B.

DISCUSSION

The results of the present study have significance in that investigations which involve the collection of serum specimens over long periods of time are dependent in some measure upon the reliability of the results of serologic tests

performed on stored sera. Since –20° C. is a readily obtainable temperature from the practical point of view, the data indicate a practical solution to the problem of storage of one serologically active substance which is labile at 4° C.[1-8] The results probably cannot be interpreted as indicating that all antibodies will not decrease in titer in sera stored at –20° C., especially since cold hemagglutinins differ in certain respects from other antibodies.

The original titers that were employed as the basis for comparison with titers obtained after storage at –20° C. were determined after the sera had been frozen at least once. Comparative data on titers of cold hemagglutinins in fresh and frozen sera were obtained only with two sera (Table II); freezing had no effect on these titers.

Variations in titers of sera tested on two or more occasions (Tables I and II) require two comments since they have pertinence in interpreting the results. Although some of the variations were extreme, the data show (*1*) that approximately 80 per cent of the titers in subsequent tests were within the limits of a fourfold (two tube) dilution of the original titer, and (*2*) that increases in titer were observed as frequently as decreases and both were of approximately equal magnitude. These observations support the conclusion that the changes in titer were due to variables in the technique rather than to quantitative changes in cold hemagglutinins.

CONCLUSION

Human sera may be stored in the frozen state (–20° C.) for at least three years without appreciable loss of titer of cold hemagglutinins.

The technical assistance of Miss Patricia Harmstead and Miss Naomi Beuhler is gratefully acknowledged.

REFERENCES

1. Peterson, O. L., Ham, T. H., and Finland, M.: Cold Agglutinins (Autohemagglutinins) in Primary Atypical Pneumonia, Science **97**: 167, 1943.
2. Horstmann, D. M., and Tatlock, H.: Cold Agglutinins: A Diagnostic Aid in Certain Types of Primary Atypical Pneumonia, J. A. M. A. **122**: 369, 1943.
3. Meikeljohn, G.: The Cold Agglutination Test in Primary Atypical Pneumonia, Proc. Soc. Exper. Biol. & Med. **54**: 181, 1943.
4. Favour, C. B.: Autohemagglutinins—"Cold Agglutinins," J. Clin. Investigation **23**: 891, 1944.
5. McNeil, C.: The Relationship of Cold Agglutinins to the Course of Primary Atypical Pneumonia, Am. J. M. Sc. **209**: 48, 1945.
6. Finland, M., and Barnes, M. W.: Cold Agglutinins. V. Deterioration of Cold Isohemagglutinins on Storage, J. Clin. Investigation **24**: 490, 1945.
7. Erf, L. A.: A Note on the Stability of Cold Hemagglutinins, Am. J. Clin. Path. **15**: 210, 1945.
8. Young, L. E.: The Clinical Significance of Cold Hemagglutinins, Am. J. M. Sc. **211**: 23, 1946.
9. Commission on Acute Respiratory Diseases: Cold Hemagglutinins in Primary Atypical Pneumonia and Other Respiratory Infections, Am. J. M. Sc. **208**: 742, 1944.
10. Feller, A. E.: Primary Atypical Pneumonia, Diagnostic Procedures for Virus and Rickettsial Diseases, ed. 1, New York, 1948, American Public Health Association.

HEMOLYSIS OF RED BLOOD CELLS TREATED BY BACTERIAL FILTRATES IN THE PRESENCE OF SERUM AND COMPLEMENT

LOWELL A. RANTZ, M.D., ANN ZUCKERMAN, A.B., AND ELIZABETH RANDALL, M.A.
SAN FRANCISCO, CALIF.

MIDDLEBROOK and Dubos[1] have described a hemagglutination reaction between the sera of tuberculous experimental animals or human beings and sheep erythrocytes treated with extracts or products of the culture filtrates of tubercle bacilli. More recently, Middlebrook[2] has demonstrated that the addition of complement to the system will cause the sensitized red cells to hemolyze in the presence of antibody.

It was obviously of great interest to extend these observations to include other organisms. Group A hemolytic streptococci were chosen for this purpose initially, and it was demonstrated that culture filtrates of these organisms were able to prepare sheep cells for hemolysis in the presence of certain human sera and complement. Later the reaction was shown to be nonspecific, the sensitizing substance being present in the filtrate of many gram-positive and occasional gram-negative bacteria.

METHODS

Thirteen strains of Group A hemolytic streptococci were studied. Ten had been isolated from clinical sources and had been preserved in the frozen state at -20° C. until used. They were selected as of the specific precipitin types 1, 5, 6, 12, 28, 29, 33, 41, 43, and 47. One was the Richards strain, and two were not typable. The latter were freshly isolated from human sources. One, Banks, was not mucoid, and produced very large amounts of streptolysin O in vitro when grown in Todd-Hewitt broth. The other, Espinoza, was persistently mucoid even after many transfers on artificial media, and produced no demonstrable antistreptolysin O in vitro.

All of the detailed studies of the phenomena to be described were performed with the filtrates of the last two strains of streptococci. A number of organisms of other species were used. All of these were freshly isolated from human sources and were identified by the usual bacteriologic methods.

The medium used for the preparation of filtrates was hog liver-stomach digest broth at pH 7.2. The dextrose content was about 0.1 per cent. The organisms to be studied were inoculated to either 200 or 1,000 ml. of this medium and incubated for ninety-six hours as 37° C. After incubation the bacteria were removed by Seitz filtration if the volume of culture was one liter. Smaller volumes were clarified by centrifugation for thirty minutes or more at 3,000 r.p.m. This was done since it was discovered that substantial amounts of the active principal were retained by the Seitz filter pad.

After filtration or clarification, the pH was adjusted to 7.0 ± 0.1, and the filtrates were distributed in 10 ml. amounts in glass tubes and stored in the frozen state at -20° C. They were thawed at 37° C. in a water bath just before use.

From the Department of Medicine, Stanford University School of Medicine.
Supported by a research grant from the National Heart Institute of the National Institutes of Health, United States Public Health Service.
Received for publication, Nov. 5, 1951.

TABLE I. HEMOLYSIS OF SHEEP RED CELLS TREATED BY EXPOSURE TO TWO DIFFERENT GROUP A HEMOLYTIC STREPTOCOCCAL FILTRATES IN THE PRESENCE OF HUMAN SERUM AND COMPLEMENT

		HEMOLYSIS OF SHEEP CELLS TREATED WITH																	
	UNTREATED SERUM DILUTION	BANKS FILTRATE SERUM DILUTION									ESPINOZA FILTRATE SERUM DILUTION								
SERUM	4	4	8	16	32	64	128	256	512	1024	4	8	16	32	64	128	256	512	1024
MS 32	0	++	0	0	0	0	0	0	0	0	+++	++	++	0	0	0	0	0	0
Zuck	0	+++	+++	+	0	0	0	0	0	0	+++	+++	+++	+++	0	0	0	0	0
MS 11	0	+++	+++	+++	+++	0	0	0	0	0	+++	+++	+++	+++	+++	+++	+++	+++	++
Bolds	0	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++

\+ – Hemolysis.
0 – No hemolysis.

Tuberculin.—Old tuberculin, four times the usual skin test potency, supplied by the Lederle Laboratories, Pearl River, N. Y., was used in certain experiments.

Complement.—Fresh Guinea pig serum pooled from twenty-five animals and stored in the frozen state was used in nearly all of the experiments as a source of complement. Lyophilized serum was occasionally utilized.

Sheep Cells.—Whole defibrinated sheep blood, not more than five days old, was used as a source of sheep cells. It was not possible to obtain sheep blood drawn directly into Alsever's solution.

Human Sera.—Human sera were collected aseptically and stored in the frozen state at -20° C. until used. Antibody titers are reported in terms of the reciprocal of the dilution of the last tube in which there was demonstrable agglutination or hemolysis. Just before use the serum was inactivated at 56° C. for thirty minutes. The antistreptolysin O titers of these sera had been determined by a method previously described.[3]

Performance of the Test.—The demonstration of hemolysis of red blood cells treated by bacterial products was accomplished by the technique described by Middlebrook.[2] No essential deviation from the methods detailed by him in Section B—I, III, IV, V, and VI—and in Section C were permitted. Minor changees in the volumes and dilution systems as suggested by Dr. Middlebrook in a personal communication were made.

Culture filtrates or dilutions of them were employed instead of tuberculin. They were always heated at 56° C. in a water bath for fifteen minutes immediately before use for the purpose of inactivating heat labile bacterial hemolysins. After exposure to the filtrates the red cells often assumed a chocolate brown color.

RESULTS

Tuberculin Sensitization of Red Cells.—The entire procedure, exactly as described by Middlebrook, was carried out using sera from known tuberculous subjects and red cells treated with tuberculin. Six sera were tested. Three had agglutinin titers of 8, one of 16, and two of 32. Hemolysins were present in all in titers of 8 in three, 16 in one, 32 in one, and 256 in one. The two sets of values did not vary in parallel.

Hemolytic Streptococci Sensitization of Red Blood Cells.—A large number of experiments were done in which filtrates of the two strains of Group A streptococci, Banks and Espinoza, were used to sensitize sheep cells which were then tested against various human sera in the presence of complement. Results of a typical experiment are presented in Table I.

These data demonstrate that a substance is present in human sera in greatly varying concentration from subject to subject which is capable of hemolyzing cells under the conditions just described. Thus the hemolysin concentration of serum MS 32 was 4 units per milliliter, that of Zuck was 16 units per milliliter, that of MS 11 was 32 units per milliliter, and that of Bolds was 1024+ units per milliliter. If complement was omitted from the system, hemolysis did not occur. Under these circumstances agglutination of the treated cells was demonstrable. The cells tended to clump spontaneously, and the determination of agglutinin titers was not satisfactory. For this reason only the hemolytic reaction was studied in detail.

The results were highly reproducible if the experimental conditions, including the potency of the complement, were rigidly controlled. The filtrates were adequately potent diluted 1:25 in phosphate buffer, but the serum titers were lower. A dilution of 1:3 was quite satisfactory and made the test easier to read, as browning of the cells was less marked.

Filtrates were prepared from ten additional strains of hemolytic streptococci of known serological types and these were used for tests with sera whose hemolysin titer had been determined against cells sensitized with Banks and Espinoza filtrates. Each of the 10 was potent and in every instance the serum titers were the same when these or the standard filtrates were used. These data are not presented in detail.

These studies demonstrated that the essential red cell sensitizing material was uniformly produced by different types of Group A streptococci.

Filtrates of a strain which formed large amounts of hyaluronic acid and very little streptolysin O (Espinoza), and another which made little of the former and very large amounts of the latter (Banks) contained comparable amounts of the critical substance.

Sensitization of Red Cells byFiltrates of Other Organisms.—No other attempts were made to characterize the red cell sensitizing substance but it was desirable to determine whether filtrates of other organisms would have a similar effect. Supernatants were prepared for this purpose by the high speed centrifugation of cultures of a variety of organisms and these were used in the test exactly as were streptococci filtrates in the experiments just described.

Three strains of *Micrococcus pyogenes*, variety *aureus*, coagulase positive, one of pneumococcus, one of nonhemolytic enterococcus, and one of *Pseudomonas sp.* all produced highly potent filtrates. Two strains of hemolytic enterococcus were also active but produced the effective agent in smaller amounts. All of these filtrates caused browning of the red cells. A strain of *Micrococcus sp.* and another of a nonhemolytic streptococcus formed a heat stable hemolysin and could not be used for testing.

Filtrates of cultures of two strains of *E. coli*, one of a Salmonella of the Kauffman-White group C_2, and one of *Proteus sp.* were completely inert and failed to sensitize the red cells. None of these extracts caused browning of the red cells.

Cells coated with the concentrated tuberculin diluted 1:4 and 1:12 failed to hemolyze in the presence of the sera which were active against cells treated with the products of streptococci and other organisms. Such cells were actively hemolyzed by sera from tuberculous human beings.

Methemoglobin Formation.—The fact that browning of the red cells occurred with potent filtrates and not with others prompted a study of the abnormal pigment. Examination of the solution of red cell contents obtained by lysing samples of treated red cells in distilled water revealed the characteristic spectroscopic absorption pattern of methemoglobin.

This substance was then formed in red cells untreated with bacterial filtrates by exposing 25 ml. of a 0.5 per cent suspension of red cells in buffer to 15 ml. of a 1 per cent solution of sodium nitrite for twenty minutes. The chemical was then removed by washing three times with buffer.

Red cells treated in this way looked exactly like those sensitized by bacterial filtrates but, when they were used in the immunologic system, no hemolysis occurred with potent sera. Methemoglobin formation per se is not responsible for the hemolysis described in this report.

Antibody Titers.—The concentration of hemolysin in the serum of healthy medical students and children, and in acute rheumatic fever were estimated by the usual procedures described above using the filtrate of Group A streptococcus Banks as the red cell sensitizing agent. For this preliminary survey titers were not determined beyond 80 units per milliliter. The results are presented in Table II.

TABLE II. SERUM HEMOLYSIN TITERS IN HEALTHY MEDICAL STUDENTS AND CHILDREN, AND IN ACUTE RHEUMATIC FEVER

SOURCE OF SERUM	NUMBER	SERUM HEMOLYTIC TITER IN UNITS PER ML.				
		<10	10	20	40	80+
Healthy medical students	55	17	13	5	10	10
Healthy children 4 to 8 years of age	18	6	5	5	2	0
Acute rheumatic fever 3 to 16 years of age	3	0	0	1	2	0

The antibody was present in measurable amounts ($>$ 10 units per milliliter) in the serum of 76.0 per cent of fifty-five healthy young adults and in large amounts (80 or more units per milliliter) in 18.2 per cent. The values in a small group of healthy children from 4 to 8 years of age and in a few cases of acute rheumatic fever were different in that the hemolysin levels were never greater than 40 units per milliliter.

There was absolutely no correlation between the concentration of sensitized sheep cell hemolysin and that of antistreptolysin O in these sera, nor with the blood groups of the subjects.

Absorption experiments were carried out in which 1.0 ml. of a 1:4 dilution of high titered sera was exposed to 0.5 ml. of packed coated and uncoated sheep cells at 3° C. for two hours. The hemolysin titers were then determined in the usual way after removal of the sheep cells by centrifugation. In a typical experiment the hemolysin titer of one serum before and after absorption with untreated sheep cells was 256. After absorption with coated cells the level fell to 32. Similarly the hemolysin concentration of another serum was reduced from 64 to 4.

DISCUSSION

The experiments described in this paper have demonstrated that bacterial filtrates are capable of sensitizing sheep red cells so that they are hemolyzed in the presence of certain human sera and complement. In the absence of complement, the sensitized cells were agglutinated. A detailed study of this phase of the problem was not made.

The effective bacterial substance was not species specific, but was produced by several gram-positive organisms, including Group A streptococcus, *Micrococcus pyogenes,* var. *aureus,* pneumococcus, enterococcus, and one gram-negative bacillus, a *Pseudomonas sp.* All other gram-negative organisms studied were inactive, including strains of *E. coli, Salmonella sp.,* and *Proteus sp.*

The nature of the sensitizing substance was not determined. It was not inactivated by exposure to 56° C. for thirty minutes, and was not present in

tuberculin used for coating cells for the Middlebrook test which demonstrates hemolysins in tuberculous serum. The reaction was demonstrated not to be the result of the formation of methemoglobin which occurred when red cells were exposed to certain filtrates.

A true immunologic process appears to be involved in the hemolysis of sensitized cells, since the phenomenon does not occur in the absence of complement. The component in serum responsible for the reaction is presumably an antibody. It is absorbed onto the coated cells, and is heat stable. The concentration of the hemolysin in the serum varies greatly from person to person.

Serum hemolysin titers were identical when the sheep cells were treated with the potent filtrates of any of several bacterial species. The antibody concentrations in a small group of children were lower than in young adults, and were low in acute rheumatic fever.

The ultimate significance of the demonstration of the hemolysis by human serum and complement of red cells sensitized by bacterial filtrates has not been determined. It is evident from these studies and those of Middlebrook that bacterial products may alter red cells and make them susceptible to damage by immunological processes. Whether these phenomena may have any role in the pathogenesis of any of the manifestations of infection, in hemolytic anemia, or in any of the disorder in which immunologic hypersensitivity may play a role remains to be decided.

Investigation of these possibilities, as well as a more elaborate investigation of the fundamental process itself, is in progress.

SUMMARY

The treatment of sheep red blood cells with filtrates of several bacterial species makes them susceptible to hemolysis by certain human sera in the presence of complement. There is a wide variation in the titer of hemolysin present in the sera from different healthy human beings, and the levels are the same regardless of the species of bacterium used in preparation of the red cell sensitizing filtrates.

REFERENCES

1. Middlebrook, G., and Dubos, R. J.: Specific Serum Agglutination of Erythrocytes Sensitized with Extracts of Tubercle Bacilli, J. Exper. Med. **88**: 521, 1948.
2. Middlebrook, G.: A Hemolytic Modification of the Hemagglutination Test for Antibodies Against Tubercle Bacillus Antigens, J. Clin. Investigation **29**: 1480, 1950.
3. Rantz, L. A., and Randall, E.: A Modification of the Technique for the Determination of the Antistreptolysin Titer, Proc. Soc. Exper. Biol. & Med. **59**: 22, 1945.

FURTHER STUDIES ON THE NATURE OF THE HEMORRHAGIC STATE IN RADIATION INJURY

D. P. Jackson, M.D.,* E. P. Cronkite, M.D.,** G. V. Leroy, M.D.,*** and Bernard Halpern, M.S.§
Bethesda, Md.

INTRODUCTION

In spite of the large amount of work that has been done on this lethal factor in ionizing radiation injury, the pathogenesis of the hemorrhagic state is still poorly understood. All investigators are agreed that there is a marked thrombocytopenia in the bleeding irradiated animal. In addition, increased capillary fragility, blood coagulation defect, and ulcerations into large blood vessels have been described as contributing to, or as the primary causes of, the fatal hemorhage. The blood coagulation defect has been considered by some[1] a result of the release of heparin or heparinlike substances, but this has been disputed by others.[2-4] Since hyperheparinemia can be satisfactorily treated by such comparatively nontoxic antiheparin substances as protamine sulfate and toluidine blue, it was considered desirable to explore further the nature of the hemorrhage produced by radiation injury with respect to the presence or absence of circulating anticoagulants. Various experimental animals have been used in the past, but it was considered desirable to use dogs in these studies to enable direct comparison with previous work.

MATERIALS AND METHODS

Experimental Animals.—Ten American male foxhounds, Walker breed, were used. The ages at the time of irradiation were between 12 and 18 months. Following irradiation, the dogs were housed in individual metabolism cages and maintained on a diet of Purina Dog Chow with a daily supplement of horse meat. Prior to irradiation, all dogs were dewormed three times with hexylresorcinol and butyl chloride. The dogs received no other treatment before or after irradiation.

Technique of Irradiation.—All of the dogs were given a total of 600 r. of 2 million volt x-rays. The irradiation was given in two doses of 300 r. delivered to each side of the body. Irradiation factors have been described.[5, 6] With this irradiation technique, the median lethal dose is 315 r. The dose of 600 r. has been uniformly fatal for more than 200 dogs. Death occurs between the sixth and fourteenth days after exposure. Two of the dogs (No. 84 and No. 85) were irradiated on July 5, 1950; the remaining eight dogs were irradiated simultaneously on July 21, 1950.

Technique for Obtaining Blood.—All venepunctures were performed by the same individual (E. P. C.), using sterile nonsiliconed 20-gauge needles. Clean, nonsterile syringes, lubricated with silicone oil,‖ were used. Venepunctures were performed on a foreleg vein with the use of a tourniquet.

From the Naval Medical Research Institute, National Naval Medical Center.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of the Navy or of the Naval Service at large.

Received for publication, Oct. 17, 1951.

*Lieutenant (jg), Medical Corps, United States Naval Reserve.
**Commander, Medical Corps, United States Navy.
***Associate Dean, Division of Biological Sciences, University of Chicago.
§Parmly Research Laboratory, Northwestern University Medical School.

‖GE Silicone Oil #9996-300.

Hematologic Studies.—The following studies were performed on venous blood three times during a control period of one week prior to irradiation, and then daily on each dog after irradiation.

(a) *Hemoglobin as oxyhemoglobin:* expressed in grams per 100 milliliters.[7]

(b) *Hematocrit:* expressed in per cent, using the technique of Wintrobe.[8]

(c) *Leukocytes:* expressed in 1,000s per cubic millimeter. Blood was diluted with 2 per cent acetic acid, using a dilution of 1 to 20 until the total count fell below 1,000 per cubic millimeter, at which time a dilution of 1 to 10 was used. The counts were performed in the usual manner.

(d) *Differential leukocyte counts* were performed on Wright-stained cover slip smears. One hundred cells were counted when possible.

(e) *Platelets:* expressed in 1,000s per cubic millimeter. Platelets were counted by the phase contrast method of Brecher and Cronkite.[9]

(f) *Whole-blood clotting time:* expressed in minutes. One milliliter of whole blood was added directly from the syringe to each of three chemically cleaned 12 mm. by 75 mm. Pyrex test tubes, immersed in a water bath at 37° C. The stopwatch was started at the time that blood was first seen in the syringe. Tubes were observed at the end of four minutes, and thereafter every thirty to sixty seconds during the next fifeen minutes. When the clotting time was in excess of twenty minutes, the period between observations of the tubes was increased. The end point was that time at which the tubes could be inverted with no flow of the surface of the blood or of the sedimented red cells. The time is recorded in minutes for the longest of the three tubes.

(g) *Clot retraction and lysis of the clot* were observed in one of the tubes in which the whole blood clotting time was determined. Observations were made at the end of twenty-four hours, during which time the tubes were maintained at 37° C. in a water bath.

(h) *Plasma fibrinogen:* expressed in grams per 100 milliliters of plasma. Isolation of fibrinogen as fibrin was performed by the technique of Cullen and Van Slyke, as modified by Hawk, Oser, and Summerson.[10]

(i) One-stage prothrombin time expressed in seconds was determined by the method described by LeRoy, Halpern, and Dolkart.[11]

Studies for the Detection of a Circulating Anticoagulant.—The following three tests were performed on the dogs during the period of controlled observation and at frequent intervals following irradiation.

(a) *Conley's technique for the detection of circulating anticoagulants:* A modification of the technique described by Conley and associates[12] was used. The test was modified as follows: (1) 20-gauge needles were substituted for the 18-gauge needls, (2) the needles were not siliconed, (3) syringes were lubricated with GE silicone oil, (4, a single-stage centrifugation was performed to remove the cellular elements and platelets, and (5) the plasma was aspirated from the sedimented cells with a silicone-oiled syringe and silicone-oiled 17-gauge needle.

Results with Conley's tests are recorded in four categories: (1) positive (the unknown platelet-poor plasma prolonged the clotting time of normal whole blood by a factor of 2 or more). (2) Questionable (the unknown plasma prolonged the clotting time of the normal whole blood slightly beyond the simultaneous control, but the prolongations of this magnitude would fall within the normal range for repeated determinations on blood from the same animal). (3) Negative (when the clotting time of the normal whole blood was not prolonged by the unknown plasma). (4) Unsatisfactory (when the unknown plasma clotted before Conley's test could be set up. Ordinarily, plasma prepared in this manner remained fluid for at least one hour. When clotting occurred in less than 30 minutes, it was attributed to technical errors).

Platelet counts on "platelet-free" plasma were performed as described above, except that a 1 to 10 dilution was used rather than the standard technique, and in addition 1 sq. mm. of the counting chamber was counted. All of the Conley type tests were performed by the same observer (D. P. J.).

TABLE I. DAILY RESULTS OF CLOTTING STUDIES ON IRRADIATED DOGS

DOG NUMBER	DAY NUMBER	CONLEY'S TEST	ALLEN'S TEST	PROTAMINE-HEPARIN RATIO	CLOTTING TIME (MIN.)	PLATELETS × 10^3/ CU. MM.
84	Control	Neg.	0.12		12.5	330
						268
						230
	1				14.0	240
	2	Unsatisfactory			22.0	320
	3				22.0	210
	4	Neg.			17.0	250
	5				18.0	220
	6	Neg.			30.0	194
	7	Unsatisfactory	0.10		32.0	160
	8	Neg.	0.12		35.0	059
	9	Neg.	0.10		28.0	030
	10	Neg.	0.10		>2 hr.	0.326
	11	Neg.	0.12		60.0	0.002
	12	Pos.	0.14		>6 hr.	0.000
Death—12th day						
85	Control		0.10		11.5	450
					11.5	300
					15.0	292
	1				18.0	330
	2	Neg.			25.0	250
	3				22.0	250
	4	Unsatisfactory			14.0	262
	5	Neg.			18.0	275
	6				32.0	240
	7	Neg.	0.12		29.0	130
	8	Neg.	0.12		37.0	090
	9	Neg.	0.10		84.0	012
	10	Questionable	0.12		>2 hr.	0.000
Death—10th day						
86	Control	Neg.	0.10	1.75/1	6.0	250
				1.75/1	13.5	370
				1.75/1	8.0	280
	1				20.0	
	2				20.0	235
	3			1.75/1	18.0	235
	4	Unsatisfactory		1.75/1	18.5	345
	5	Neg.		1.75/1	25.0	255
	6		0.12	1.75/1	29.0	245
	7	Neg.	0.10	1.75/1	34.5	165
	8	Neg.	0.12	1.75/1	48.0	085
	9	Unsatisfactory		1.75/1	76.0	015
	10	Neg.	0.14	1.75/1	6 hr.	003
	11	Neg.	0.14	1.75/1	9 hr.	003
Death—11th day						
89	Control	Neg.	0.10	1.75/1	13.0	360
				1.75/1	12.0	350
					18.0	345
	1				19.0	
	2				16.0	305
	3			1.75/1	21.0	350
	4	Unsatisfactory		1.75/1	17.5	410
	5	Neg.		1.75/1	25.5	260
	6		0.12	1.75/1	22.0	265
	7	Neg.	0.12	1.75/1	36.0	125
	8	Neg.	0.12	1.75/1	60.0	065
Death—8th day						

TABLE I—CONT'D

DOG NUMBER	DAY NUMBER	CONLEY'S TEST	ALLEN'S TEST	PROTAMINE-HEPARIN RATIO	CLOTTING TIME (MIN.)	PLATELETS $\times 10^3$/CU. MM.
90	Control	Neg.	0.10	1.75/1	5.0	180
				1.75/1	16.0	290
					14.0	285
	1				20.0	
	2				14.0	230
	3			1.75/1	14.0	310
	4	Unsatisfactory		1.75/1	20.0	410
	5	Neg.		2.625/1	28.0	265
	6		0.12	1.75/1	30.0	215
	7	Neg.	0.14	1.75/1	54.0	120
	8	Neg.	0.12	1.75/1	68.5	010
	9	Neg. 30 min.	>0.16	1.75/1	>24 hr.	0.000
Death—9th day			Post mortem			
91	Control	Neg.	0.10	1.75/1	11.0	350
				1.75/1	21.0	260
				1.75/1	18.0	320
	1				19.0	
	2				16.0	345
	3			1.75/1	14.5	255
	4	Unsatisfactory		1.75/1	17.0	210
	5	Pos.		2.625/1	21.0	215
	6		0.12	1.75/1	27.0	270
	7	Unsatisfactory	0.12	1.75/1	48.0	120
	8	Neg.	0.12	1.75/1	28.0	115
	9	Neg.	0.12	1.75/1	58.0	045
	10	Unsatisfactory	0.14	1.75/1	86.5	020
	11	Neg.	0.16	1.75/1	84.0	0.000
	12	Neg.	>0.18	1.75/1	>9 hr.	0.000
	13	Neg.			>9 hr.	0.001
Death—13th day						
92	Control	Pos.		1.75/1	16.0	280
		Neg.	0.10	1.75/1	14.0	270
		Neg.		1.75/1	14.5	250
		Neg.				
	1				16.0	
	2				13.0	390
	3			1.75/1	16.0	385
	4	Questionable		1.75/1	17.0	370
	5			1.75/1	19.0	340
	6	Unsatisfactory	0.10	1.75/1	33.0	205
	7		0.12	1.75/1	54.0	155
	8	Neg.	0.12	1.75/1	17.0	110
	9	Neg.	0.10	1.75/1	59.5	075
	10	Neg.	0.16	1.75/1	120.0	010
	11	Neg.	>0.18	1.75/1	>9 hr.	0.000
	12	Neg.	>0.18	1.75/1	>9 hr.	0.000
Death—12th day						
				1.75/1	17.0	150
93	Control	Neg.	0.12	1.75/1	10.0	200
					14.0	315
	1				17.0	
	2				19.0	270
	3			1.75/1	14.0	360
	4	Neg.		1.75/1	20.0	345
	5			1.75/1	14.0	300
	6	Unsatisfactory	0.14	1.75/1	28.5	335
	7		0.14	1.75/1	44.0	200
	8	Questionable	0.12	1.75/1	37.0	090
	9	Pos. (?)		1.75/1	83.0	050
	10	Neg.	0.14	1.75/1	113.0	006
	11	Neg.	0.14	1.75/1	173.0	0.000
Death—12th day						

TABLE I—CONT'D

DOG NUMBER	DAY NUMBER	CONLEY'S TEST	ALLEN'S TEST	PROTAMINE-HEPARIN RATIO	CLOTTING TIME (MIN.)	PLATELETS $\times 10^3$/CU. MM.
				1.75/1	15.0	190
106	Control	Neg.	0.12	1.75/1	10.0	200
				1.75/1	13.0	390
	1					
	2				12.5	220
	3			1.75/1	14.5	310
	4	Neg.		1.75/1	21.0	305
	5			1.75/1	22.0	250
	6	Neg.	0.12	1.75/1	35.0	135
	7		0.14	1.75/1	48.5	125
	8	Neg.	0.14	1.75/1	58.0	080
	9	Neg.	0.16	1.75/1	101.0	030
	10	Neg.	>0.16	1.75/1	>6 hr.	001
Death—10th day						
				1.75/1	17.0	420
108	Control	Neg.	0.10	1.75/1	10.0	300
				1.75/1	18.5	300
	1					
	2				16.0	395
	3			1.75/1	16.5	375
	4	Neg.		1.75/1	21.0	300
	5			1.75/1	27.0	270
	6	Neg.	0.14	1.75/1	26.0	205
	7		0.14	1.75/1	40.0	130
	8	Neg.	0.16	1.75/1	55.0	055
	9	Neg.	0.16	1.75/1	>8 hr.	003
	10	Questionable	>0.16	1.75/1	>6 hr.	0.000
Death—10th day						

(b) *Allen's whole blood protamine-heparin titration:* A modification of the technique described by Allen and co-workers[13] was used. The points at which the technique was modified were: (1) a 20-gauge needle was used, (2) powdered Connaught heparin was used from which a solution containing 5 mg. of heparin per milliliter of normal saline was prepared, (3) 5.5 ml. of blood were added to a conical graduated centrifuge tube containing 0.1 ml. of this heparin solution (i.e., 0.5 mg. of heparin), and (4) a series of 5 (rather than 10) tubes containing increasing amounts of protamine sulfate ranging from 0.08 to 0.16 mg., or 0.10 to 0.20 mg. were set up. Allen's test was said to be abnormal when the amount of protamine sulfate required to clot heparinized whole blood was 0.16 mg. or more.

The same observer (B. H.) performed all of these tests, and, on most occasions, a normal, nonirradiated control animal was run simultaneously with the postirradiated animals.

(c) *LeRoy's heparin-protamine titration test,* using oxalated plasma, was performed in the manner described by LeRoy, Halpern, and Dolkart.[11] The test was said to be abnormal when the ratio of protamine to heparin at which the fastest one-stage prothrombin time occurred was greater than 1.75 to 1. The same observer (B. H.) performed all of these tests.

RESULTS

Clinical Observations.—Purpura of the oral mucous membranes and the skin appeared in all of the dogs prior to death. The earliest onset of purpura was the seventh day, and all dogs had evidence of purpura by the tenth day after irradiation. In general, purpura was more severe in the dogs that lived the longest. Other manifestations of a hemorrhagic state, such as melena and hematuria, occurred in these animals. At autopsy all showed extensive evidence of diffuse hemorrhage throughout their organs, with the exception of the brain.

One dog had a small cerebral hemorrhage. The gross and microscopic pathologic effects of radiation which occurred were similar to the lesions that have been described amply by others.[14-16] From the fourth day before death until death there was a rapid increase of the body temperature, and, on the last day of life, the mean temperature was 105.5° F. Death occurred between the eighth and thirteenth day.

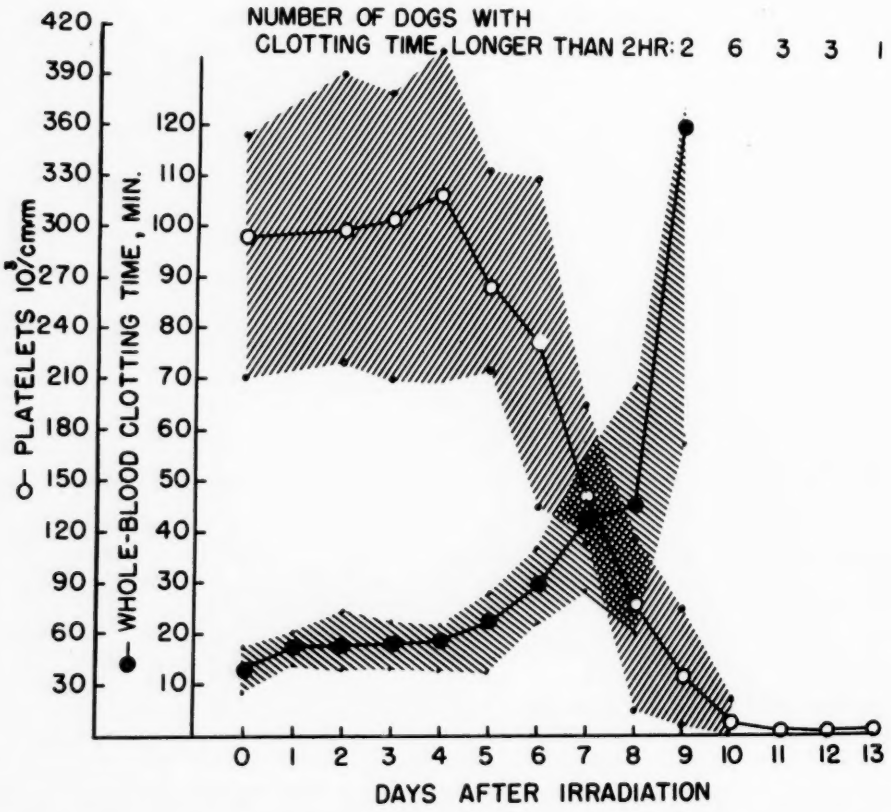


Fig. 1.—Mean values for whole-blood clotting time and platelet count after irradiation. The shaded envelopes include the range of the daily values.

Hematologic Studies.—After irradiation, the decrease in the hemoglobin, hematocrit, and leukocyte count occurred in the manner which is typical for dogs exposed to such a dose of x-rays. As an average, the leukocyte count was 1,000 or less from the sixth day on. In Fig. 1 are plotted the daily mean platelet counts and the mean whole-blood clotting times, along with the range, for the ten dogs. From the figure it can be seen that during the first four days following irradiation the platelets did not decrease.* Thereafter, the platelets de-

*In our experience there is considerable variation in the platelet count during the first four days after exposure to a lethal dose of radiation. The usual result is either no significant change, or a slight decrease. Other workers have reported decreased values from the day of irradiation. Since the methods of counting vary, it is difficult to account for the discrepancies.

creased rapidly until they reached nearly zero by the tenth day after irradiation. Beginning on the sixth day there was a progressive increase in the whole-blood clotting time. From Fig. 1 there appears to be a relation between the fall of the platelets and the rise of the whole-blood clotting time.

Studies of Blood Coagulation.—

(1) *Whole-blood clotting time:* The whole-blood clotting time remained within normal limits for the first five days (Fig. 1). The first significant increase in the mean clotting time occurred on the sixth day after irradiation, and

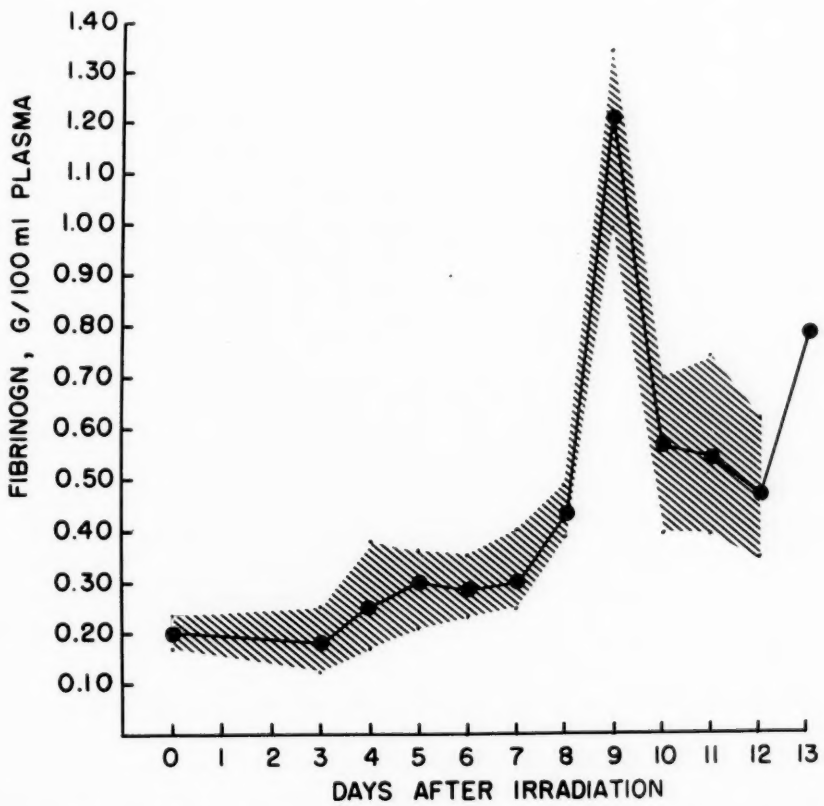


Fig. 2.—Mean values and range of the plasma fibrinogen concentration after irradiation.

thereafter the mean and range showed a steady increase. Each of the ten dogs used in this experiment had a whole-blood clotting time of one hour or longer by the day of death, and five of the ten dogs had clotting time of one hour or longer for two days or more preceding the day of death.

(2) *Twenty-four hour clot retraction:* Complete absence of clot retraction at twenty-four hours was observed in all of the ten dogs at some time prior to death. In those dogs surviving nine days or longer, no retraction was observed after the eighth postirradiation day.

(3) *Twenty-four hour lysis of clot:* Lysis of the clot at the end of twenty-four hours was observed in seven of the ten dogs on the last day of life.

(4) *Plasma fibrinogen:* In Fig. 2, the mean value and the range of the plasma fibrinogen are plotted. Beginning on the fourth day there was a slow increase in the fibrinogen concentration. From the eighth day until death, the fibrinogen concentration was at least twice as high as the control levels. The nature of the clot formed in the one-stage prothrombin system manifests the increased plasma fibrinogen concentration as the clot became bulky and much firmer.

(5) *One-stage prothrombin time* was performed daily on each dog (total of sixty-seven tests) as a part of the heparin-protamine titration test using oxalated plasma. There was no significant difference between the prothrombin time before and after irradiation. The values before and after irradiation ranged from 94 to 104 per cent of normal prothrombin time.

(6) *Studies for the detection of a circulating anticoagulant:* Table I presents in tabular form, for each of the ten dogs, the day-by-day results of Conley's, Allen's, and LeRoy's tests, as well as the platelet counts and whole-blood clotting time. Table II presents a summation of the incidence of various results with Conley's, Allen's, and LeRoy's tests that were performed during the postirradiation period.

(a) *Conley's Technique for the Detection of Circulating Anticoagulants:* The daily results of Conley's test are given in Table I. An unexplained observation was the occurrence of a positive test on dog No. 92 during the control period. However, three subsequent control tests, as well as all of the post-irradiation Conley's tests on this dog, were negative (see Table I). No explanation for the occurrence of this positive test during the control studies is known.

In summary, following 600 r. total body irradiation, the results of Conley's test for the detection of circulating anticoagulants were as follows for the ten dogs:

1. The test remained *negative* throughout in (Nos. 86, 89, 90, and 106)	4 dogs
2. *Questionably positive* tests on the last day of life (tenth postirradiation day) in (Nos. 85 and 108)	2 dogs
3. *Questionably* positive test on the fourth post-irradiation day only, in (No. 92)	1 dog
4. *Questionably positive* tests on the eighth and ninth days (same dog) only, in (No. 93)	1 dog
5. Test *positive* on fifth postirradiation day only, in (No. 91)	1 dog
6. Test *positive* on the last day of life (twelfth post-irradiation day) only, in (No. 84)	1 dog
Total	10 dogs

(b) *Allen's Whole Blood Protamine-Heparin Titration:* The normal range for this test in our laboratory for normal dogs was 0.10 to 0.14 mg. protamine

sulfate (Table I). In summary, following 600 r. total-body irradiation, the results of Allen's test were as follows:

1. The test remained within the *normal* range in (Nos. 84, 85, 86, 89, and 93)	5 dogs
2. *Abnormal* test on a sample of blood obtained thirty minutes post mortem in (No. 90)	1 dog
3. *Abnormal* test on the last two days of life in (Nos. 91 and 106)	2 dogs
4. *Abnormal* test on the last three days of life in (Nos. 92 and 108)	2 dogs
Total	10 dogs

(c) *LeRoy's Heparin-Protamine Titration of Oxalated Plasma:* In the control studies on normal dogs, the ratio or protamine to heparin at which the fastest one-stage prothrombin time was obtained was invariably 1.75 to 1

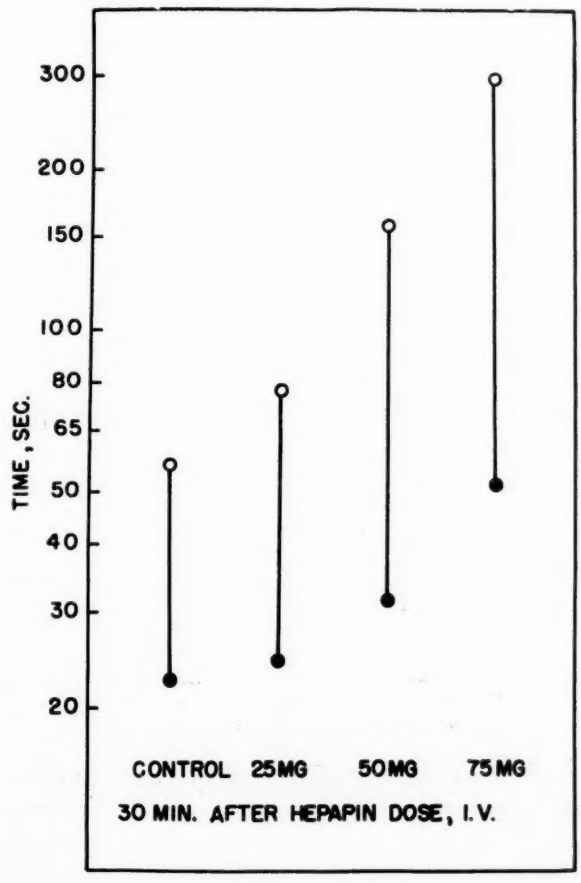


Fig. 3.—The effect in normal dogs of increasing doses of heparin, administered intravenously, on the clotting time of oxalated plasma (solid circles), and on the clotting time of oxalated plasma to which heparin (10 μg per milliliter) has been added in vitro (open circles). The addition of an appropriate amount of protamine to any of the treated plasmas would reduce the clotting time to that of the control sample (solid circle).

(Table I). Following 600 r. total-body irradiation, the following results were obtained in 8 dogs:

1. *Normal* ratios daily in (Nos. 86, 89, 92, 93, 106 and 108)	6 dogs
2. *Abnormal* ratio on the 5th day, *normal* thereafter in (Nos. 90 and 91)	2 dogs
Total	8 dogs

When this heparin-protamine titration test is performed on oxalated plasma of dogs injected with increasing amounts of heparin, a characteristic result is obtained which is illustrated in Fig. 3. The vertical lines demonstrate the increased sensitivity of such plasma to the small amount of heparin added in vitro for the test. The results obtained from a typical irradiated dog are displayed in a similar manner in Fig. 4. It is apparent that the oxalated plasma from this animal did not exhibit any significant change in sensitivity to heparin added in vitro. The experimental results, therefore, are clearly incompatible with an actual increase in the concentration of heparin in the plasma of the irradiated dogs.

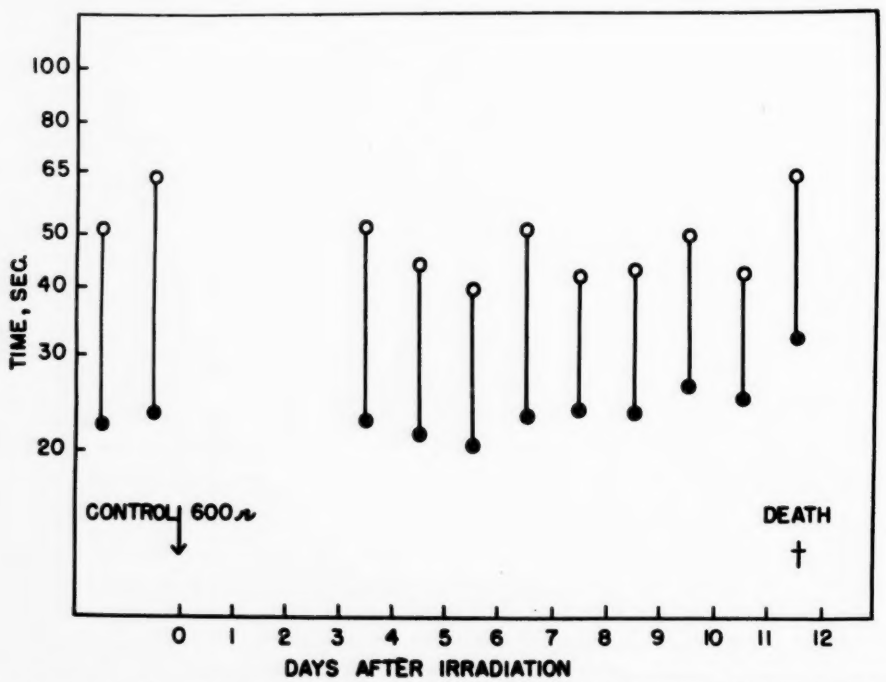


Fig. 4.—The effect of the addition of heparin (10 μg per milliliter) in vitro to samples of oxalated plasma obtained from dog 86 before and after 600 r. of whole-body x-irradiation. The daily variations in the control one-stage clotting time (solid circles) and the clotting time of oxalated plasma plus heparin (open circles) do not differ significantly from the control tests made on the same dag.

DISCUSSION

A hemorrhagic state associated with defective coagulation of blood can be attributed to a deficiency of one or several of the substances required for clotting, or to the presence of one or several substances which can inhibit clotting, or to some combination of these conditions. From the standpoint of practical therapy,

TABLE II. RESULTS OF TESTS FOR PRESENCE OF AN ANTICOAGULANT BEFORE AND AFTER X-IRRADIATION

	CONLEY'S TEST				PROTAMINE: HEPARIN RATIO		ALLEN'S TEST MG. PROTAMINE SULFATE						
	POSITIVE	QUESTIONABLE	NEGATIVE	UNSATISFACTORY	1.75/1	OTHER	0.10	0.12	0.14	0.16	>0.16	0.18	>0.18
Controls	1	0	11	-	21	0	7	6	4				
After 600 r. x-rays	2	5	45	12	65	2	7	20	13	5	3	0	3

it is of the utmost importance to ascertain the cause of defective coagulation of the blood resulting from radiation injury. In the present study three special techniques for the detection of circulating anticoagulants were used. The results are summarized in Table II. Conley's test requires the preparation of "platelet-free" or "platelet-poor" plasma, which is added to freshly-drawn normal whole blood, the clotting time of which is then measured. It is a sensitive system, and one which permits the demonstration of the effect of small amounts of an anticoagulant, either added to the plasma or naturally present in it. It is not possible with Conley's test to determine the nature of such anticoagulants as may be detected. Since normal whole blood is the indicator used, a certain variability in the response is inevitable. In the present study, Conley's test was performed successfully fifty-two times, and was certainly positive only twice.

The heparin-protamine titration tests of Allen and LeRoy depend on a curious phenomenon which is not understood. When normal blood is treated with a specified quantity of heparin, a definite amount of protamine sulfate will restore the clotting time to normal. The ratio of the protamine required to the heparin added appears to be constant for normal whole blood or normal oxalated plasma. When blood which is hypocoagulable is treated in this way the amount of protamine required will exceed the normal ratio, if the abnormal clotting is due to heparin or heparinlike material. In the case of blood obtained from patients under treatment with heparin, a characteristic increase in the protamine required (i.e., in the ratio) is found. Using whole blood (Allen's method) fifty-one successful tests were performed on the group of irradiated dogs, eleven of which were abnormal. In five of the ten dogs, the tests were normal on every occasion. It is of interest to report that on many occasions when the clotting time of whole blood exceeded one hour, samples of such blood treated with protamine in the Allen test had clotted in less than one hour. The significance of this observation is not known.

The heparin-protamine phenomenon also was studied using oxalated plasma with a one-stage prothrombin time test as the indicator (method of LeRoy, Halpern, and Dolkart). This technique is somewhat more reproducible than that using whole blood, and the combining ratio of protamine and heparin is constant. In the group of irradiated dogs this test was performed sixty-seven times,

and was abnormal on one occasion in each of two dogs. The entire pattern of the behavior of oxalated plasma from irradiated dogs with abnormal bleeding was entirely unlike that of plasma from dogs to whom heparin had been given.

Although an occasional single test suggested that the defectively coagulating blood, postirradiation, contained a circulating anticoagulant, there was no consistency in the results of the three tests used. On no occasion were all three tests abnormal on the same day, and on only one occasion were two of the three abnormal at the same time. It seems proper to consider the occasional abnormal result as a laboratory error, and not as evidence of the sporadic occurrence of an anticoagulant. Nevertheless, it is entirely possible that an anticoagulant of some type may occur sporadically. In this connection it should be noted again that when Allen's test was performed with hypocoagulable blood, the addition of protamine in vitro regularly reduced the clotting time toward normal, in spite of the fact that heparin had been added previously. This cannot be attributed simply to the effects of handling the whole blood, since a comparable result has been observed when protamine or toluidine blue is given intravenously. It appears that the action of these antiheparin agents remains to be explained.

It was regularly observed at autopsy that the blood clotted firmly within a few minutes of the initial incision. This observation is not surprising, in view of the elevated concentration of fibrinogen which was found commencing on the sixth day after irradiation. The determinations of prothrombin time, using the one-stage method, were normal on every occasion. The method used an excess of thromboplastin in order that the clotting time of oxalated plasma should be a convenient value. It is unlikely that any of these methods were sufficiently sensitive to demonstrate the existence of antithromboplastins.

The principle, obvious defect in the clotting system of these irradiated dogs was the thrombocytopenia. Since the results showed an increase of fibrinogen, an adequate concentration of prothrombin, and inconclusive evidence of a circulating anticoagulant, it appears that the abnormal clotting was caused by the decreased number of platelets resulting from radiation injury to the bone marrow.

Since these studies were made on dogs of the same breed, irradiated simultaneously, and subjected to identical procedures, the results should be considered significant. Any study of abnormal clotting of blood is attended by uncertainty due to technical conditions which are often difficult to control. It is possible to reduce this technical uncertainty by simultaneous treatment of the subjects, and simultaneous analysis of the material. In the present study each operation was performed by the same observer, at the same time of day, and all the tests on the group of animals were performed with the same batches of reagents. Under these conditions, the negative evidence for the existence of a circulating anticoagulant is impressive.

SUMMARY AND CONCLUSIONS

1. The hemorrhagic state ocurring in severe radiation injury was studied in ten dogs exposed to 600 r. of whole-body x-radiation.

2. The occurrence of abnormal coagulation of the blood, spontaneous purpura and thrombocytopenia were closely related.

3. No conclusive evidence was found for the presence of a circulating anticoagulant of the type that could be detected by Conley's test, or by heparin-protamine titration tests.

4. Specific tests for antithromboblastins and antithrombins were not performed.

5. It appeared that the clotting defect, postirradiation, was due to the decrease in the number of circulating platelets.

The authors wish to acknowledge the technical assistance of Chief Hospitalman D. C. Eltzholtz, U.S.N.; Chief Hospitalman P. K. Schork, U.S.N.; Chief Hospitalman C. R. Sipe, U.S.N.; Chief Hospitalman J. H. Pierce, U.S.N.; Miss Mildred Waters, Biological Aide. The cooperation of the Radiation Technology Division, Naval Medical Research Institute, and Commander F. W. Chambers, Jr. (M.S.C.) U.S.N., and Commander Jasper Morgan, (M.S.C.) U.S.N.; and of the Radiology Division of the Naval Ordnance Laboratory, White Oaks, Md., is greatly appreciated.

REFERENCES

1. Allen, J. G., Sanderson, M., Milham, M., Kirschon, A., and Jacobson, L. O.: Heparinemia (?) An Anticoagulant in the Blood of Dogs with Hemorrhagic Tendency after Total Body Exposure to Roentgen Rays, J. Exper. Med. **87**: 71-86, 1948.
2. Cronkite, E. P.: The Hemorrhagic Syndrome of Acute Ionizing Radiation Illness Produced in Goats and Swine by Exposure to the Atomic Bomb at Bikini, 1946, Blood **5**: 32-45, 1950.
3. Holden, W. D., Cole, J. W., Portmann, A. F., and Storaasli, J. P.: Hypothromboplastinemia Following Total Body Irradiation, Proc. Soc. Exper. Biol. & Med. **70**: 553-556, 1949.
4. Rosenthal, R. L., and Benedek, A., II: Biological Studies of Radiation Effects; Effects of Total Body X-irradiation on Blood Coagulation in the Rabbit, U.S.A.E.C. Document, AECU-592 (UCRL-414), Feb. 22, 1950.
5. Chapman, W. H., Cronkite, E. P., Chambers, F. W., Jr., and Morgan, J. E.: Experimental Procedures for the Simultaneous Exposure of Large Numbers of Animals to Total Body X-radiation, NMRI Project #NM 006 012.05.03, July 3, 1950.
6. Chambers, F. W., Jr., Morgan, J. E., and Istock, J. T.: Output Characteristics of a Commercial X-ray Generator at 2000 KVP, NMRI Project #NM 006 012.08.26 (formerly #NM 007 039), Dec. 7, 1949.
7. Notes on Operation of the Evelyn Photoelectric Colorimeter, Rubicon Electrical Instrument Makers, Philadelphia, Pa., p. 34, September, 1943.
8. Wintrobe, M. M.: Clinical Hematology, ed. 2, Philadelphia, 1946, Lea and Febiger, p. 242.
9. Brecher, G., and Cronkite, E. P.: Morphology and Enumeration of Human Blood Platelets, J. Applied Physiol. **3**: 365-377, 1950.
10. Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry, ed. 12, Philadelphia and Toronto, 1947, The Blakiston Co., pp. 548-550.
11. LeRoy, G. V., Halpern, B., and Dolkart, R. E.: An Indirect, Quantitative Method for the Estimation of Heparin Activity In Vitro: The Heparin-Protamine Titration Test, J. Lab. & Clin. Med. **35**: 446-458, 1950.
12. Conley, C. L., Hartmann, R. C., and Morse, W. I., II: Circulating Anticoagulants: A Technique for Their Detection and Clinical Studies, Bull. Johns Hopkins Hosp. **84**: 255-268, 1949.
13. Allen, J. G., Moulder, P. V., Elgehammer, R. M., Grossman, B. J., McKeen, C. L., Sanderson, M., Egner, W., and Crosbie, J. M.: A Protamine Titration as an Indication of a Clotting Defect in Certain Hemorrhagic States, J. Lab. & Clin. Med. **34**: 473-476, 1949.
14. Warren, S.: Effects of Radiation on Normal Tissues, Arch. Path. **34**: 443; 562; 749; 917; 1070, 1942.
15. Tullis, J. L.: The Response of Tissue to Total Body Irradiation, Am. J. Path. **25**: 829-852, 1949.
16. Liebow, A. A., Warren, S., and DeCoursey, E.: Pathology of Atomic Bomb Casualties, Am. J. Path. **25**: 853-1028, 1949.

THE TREATMENT OF POSTIRRADIATION INFECTION WITH ANTIBIOTICS; AN EXPERIMENTAL STUDY ON MICE

C. Phillip Miller, M.D., Carolyn W. Hammond, Ph.D.,
Marianne Tompkins, B.S., and Gertrude Shorter
Chicago, Ill.

PREVIOUS reports[1, 2] from this laboratory have described experiments which demonstrated the occurrence of bacteremia in a large proportion of mice during the second and third weeks after their exposure to 450 or 600 r total body x-radiation. The bacteremia was caused by microorganisms normally present in the lower intestinal tract. Since this generalized infection developed during the period of greatest mortality the conclusion was drawn that infection plays a significant role in death from radiation injury. The following experiments support the validity of that conclusion by showing that the administration of suitable antibiotics effectively controls such infection in irradiated mice.*

Howland and associates[6] have reported that the administration of aureomycin prolonged the lives of rats and dogs irradiated with lethal doses of x-ray, and that the drug was more effective than chloramphenicol, streptomycin, sulfadiazine, or penicillin. In a later experiment, however,[7] aureomycin failed to reduce the mortality of irradiated rats, but did lessen the incidence of diarrhea. Furth and Coulter[8] have reported that death occurred in only two of five irradiated dogs treated with aureomycin, as compared with four of five controls.

In a larger series of dogs, Allen, Moulder, and Enerson[9] found that aureomycin prolonged life and lessened the weight loss which occurs after irradiation.

Cronkite[10] administered penicillin to some of the goats exposed to ionizing radiation at the atomic bomb tests at Bikini. His data suggested that some benefit may have resulted from this treatment, but the lack of an adequate number of controls prevented him from drawing any definite conclusions. More recently Cronkite and Jackson[11] found that three of four dogs treated with penicillin and aureomycin survived irradiation with 400 r, a dose which killed 89 per cent of nineteen untreated dogs. Antibiotic therapy was not begun until ulceration and fever had developed.

Koletsky and Christie[12] found that treatment with streptomycin and penicillin in combination effectively reduced the thirty-day mortality of rats injected with radioactive phosphorus.

METHODS

Mice.—During the course of these experiments, which have extended over a period of two years, three strains of mice were used. The experiments were

From the Department of Medicine and the Institute of Radiobiology and Biophysics, The University of Chicago.

This investigation was carried out under a contract between the U. S. Atomic Energy Commission and the University of Chicago.

Received for publication, Sept. 21, 1951.

*Preliminary reports have already appeared.[3-5]

begun in May, 1949, with Maple Grove Swiss mice. In April, 1950, these mice began to show heavy infestations with mouse tapeworm, and their use was discontinued. Carworth CF #1 mice were substituted and used until October, 1950, when the incidence of infection with *Salmonella typhimurium* reached troublesome proportions. Since that time the RAP strain of Rockland Farms' mice has been used.

All of the Maple Grove mice were young adult males. The age of these animals was not known; at the time they were irradiated they weighed from 18 to 22 grams, with an average of 20 grams. The Carworth CF #1 and the Rockland mice were young adult females ten weeks old at the time they were x-rayed.

All mice were held in a quarantine room for one week before irradiation, after which they were transferred to a second room. The temperature of both rooms was maintained at 73° to 78° F. The humidity was not controlled.

During the period of quarantine the mice were housed in sheet metal cages measuring 13 by 18 by 8 inches. After irradiation they were usually kept in stainless steel cages measuring 14 by 10 by 6½ inches.

The floors were covered with wood shavings. The cages were changed every week and sterilized. The numbers of mice confined in the larger cages never exceeded 40, and in the smaller cages 15 to 20.

Diet.—Pellets of ground food were fed to mice which received subcutaneous injections. In the first few experiments Purina Dog Checkers were used; in all of the rest, mouse pellets made by Derwood Mills according to a formula prescribed by the Argonne National Laboratory were used.

In experiments involving the oral administration of drugs, the mice were fed on ground food made by Derwood Mills, which contained the same ingredients as the pellets. It was made available to the mice in metal feeding troughs, provided with covers which minimized contamination by feces and urine.* The drug was administered by incorporating it into the ground food.

Water bottles were changed every morning. The importance of daily sterilization of water bottles and drinking tubes will be discussed below.

Method of Irradiation.—All of the irradiation was carried out at the Argonne National Laboratory.† The mice were subjected to total body x-radiation in a single exposure, delivered at 250 kv., 15 ma., at a distance of 27 inches using ½ mm. copper and 3 mm. bakelite filters, at a rate of approximately 20 r per minute. Sixteen mice at a time were placed in plastic tubes measuring 3 cm. in diameter and 10 cm. in length, perforated to allow adequate ventilation. The tubes were closed with cork stoppers and fastened by elastic bands to a circular plate which rotated slowly within the irradiated field. The dose of x-ray in each experiment is indicated in the tables. It varied from 450 r to 600 r. The dosage was checked after every second run by means of a Victoreen ionization chamber.

Distribution of Mice in Experimental and Control Groups.—The mice were transported in the quarantine cages to the Argonne National Laboratory for

*We are indebted to Dr. Walsh McDermott of the Department of Medicine, Cornell University Medical College for a sample of these containers.

†We are indebted to the staff of the X-ray Laboratory of the Argonne National Laboratory for this service, which was arranged through the courtesy of Dr. Austin M. Brues, Director of the Division of Biological and Medical Research, Argonne National Laboratory.

irradiation. After their return to the mouse room, they were redistributed by putting together into each of the new cages an equal number of mice from each quarantine cage. This was done to distribute equally among the cages mice which had previously been housed together. Cages of mice were then chosen at random to serve as experimental or control groups. This insured a random distribution of animals among the two groups.

Period of Observation.—The mice were inspected several times each day. In several of the early experiments the mice were kept under observation for sixty days, but death occurred so rarely during the second month that all subsequent experiments were terminated thirty days after irradiation.

Control Mice.—Each experiment included a group of controls, i.e., untreated mice which had been exposed to the same dose of x-ray at the same time as the treated ones, and had been separated by random selection. The number of the controls usually equalled or approximated the number of treated mice and were subjected to the same conditions. Each time a group of treated animals received subcutaneous injections of drug the control mice were given subcutaneous injections of saline. When a drug was administered in ground food, the controls were fed plain ground food.

It was found to be inadvisable to mix control and treated mice in the same cage because: (a) this was impossible when drugs were given in the food; and (b) control mice could not be prevented from ingesting small amounts of drug. Mice commonly lick each others' fur when it is wetted with alcohol, and this frequently becomes contaminated with some of the drug which may leak back through the puncture wound after subcutaneous injection.

Administration of the Antibiotic.—The following antibiotics were employed alone or in combination: streptomycin, penicillin, chloramphenicol, aureomycin, terramycin, polymyxin, neomycin, and bacitracin.* Of these only streptomycin and penicillin were successfully administered by subcutaneous injection. They were injected in 0.5 ml. of saline once a day. When streptomycin was the only drug used, it was given from the fourth to the twenty-eighth day.

Chloramphenicol, aureomycin, and terramycin were given to the mice in their food. The drugs were added to the finely ground food and thoroughly mixed in an "excentric drug mixer" for a minimum of two hours.† Preliminary experiments with each of these drugs‡ showed them to be too irritating to be administered daily by subcutaneous injection. Necrosis of the skin occurred in many of the mice. Administration by gavage, i.e., introduction into the stomach through a tube, proved to be too strenuous a procedure to be repeated each day on mice suffering from radiation injury. Observations on eight mice which were housed individually in separate glass jars indicated that each ate at least 2 Gm. of ground food a day. It was on this basis that concentrations of these drugs were fixed.

*The antibiotics used in these experiments were kindly furnished by Abbott Laboratories, Commercial Solvents Corporation, Eli Lilly and Company, Lederle Laboratories, Merck & Company, Parke, Davis & Company, Chas. Pfizer and Company, Inc., E. R. Squibb & Sons.

†We are indebted to Professor E. M. K. Gieling of the Department of Pharmacology, University of Chicago, for the use of this mixer.

‡Chloramphenicol was given subcutaneously in 25 per cent propylene glycol in water, aureomycin in saline or sodium glycinate, and terramycin in saline.

TABLE I. RESULTS OF TREATMENT WITH STREPTOMYCIN BY SUBCUTANEOUS INJECTION

DATE IRRADIATED	MICE			X-RAY DOSE R	DRUG DOSE μG†	MORTALITY (30 DAY)	
	STRAIN	NUMBER					
		CONTROL*	TREATED			CONTROL	TREATED
6-20-49	MG	29	29	450	1,000	89%	96%
			29				65
6-20-49	MG	29	29	450	2,000	82	96
			29				72
Total		58	116			86%	83%
5-23-49	MG	28	32	450	3,000	64%	21%
			32				25
5-23-49	MG	28	32	450	4,000	96	25
			31				35
4- 4-49	MG	38	37	450	5,000	81	35
7-11-49	MG	40	40	450	5,000	92	37
11- 7-49	MG	24	24	450	5,000	87	25
12- 5-49	MG	20	20	450	5,000	85	35
Total		178‡	248			84%	30%
5- 9-49	MG	32	32	450	6,000	71%	12%
		32	32			75	15
11- 7-49	MG	24	24	450	6,000	87	25
11-21-49	MG	43	46	450	6,000	76	34
2- 6-50	MG	40	40	450	6,000	67	30
3- 6-50	CF#1	39	37	550	6,000§	92	16
Total		210‡	211			78%	23%
5- 9-49	MG	32	32	450	7,000	71%	59%
		32	32			75	25
11- 7-49	MG	24	24	450	7,000	87	20
12- 5-49	MG	20	20	450	7,000	85	45
2-13-50	MG	40	40	450	7,000	45	20
		40	40			32	15
Total		188‡	198			61%	24%

*Control mice injected with 0.5 ml. saline subcutaneously each day the treated mice received streptomycin.

†Dose of drug injected in 0.5 ml. saline subcutaneously once a day from the fourth through the twenty-eighth day postirradiation.

‡Including duplications.

§Treatment stopped on twenty-second day.

MG = Maple Grove Mice, Males; CF#1 = Carworth Farms #1 Mice, Females.

The method of administering drugs in the mouse's food has two disadvantages: (a) the dosage cannot be accurately measured, and (b) the mice are likely to get inadequate amounts during the second week postirradiation because they lose their appetites at that time. This is the period when they are in greatest need of medication. To compensate for this reduction in dosage, oral treatment with chloramphenicol, aureomycin, and terramycin was supplemented during the second week by daily injections of streptomycin in most experiments. In some experiments supplementary treatment with streptomycin was also given during the first four days in the hope that the development of streptomycin resistance might render the indigenous bacterial flora more sensitive to the action of the other drug, as reported by Herrell, Heilmann, and Wellman.[13]

Autopsy Cultures.—During the past year (May, 1950, to June, 1951) cultures were made on as many mice as could be autopsied immediately after death. This was done to determine whether or not bacteremia was present in the mice which died, and to identify the organisms cultured from the blood stream.

TABLE II. RESULTS OF TREATMENT WITH PENICILLIN AND STREPTOMYCIN SUBCUTANEOUSLY

DATE IRRADIATED	MICE			X-RAY DOSE R	PENICILLIN AMT. UNITS*	STREPTOMYCIN AMT. μG*	MORTALITY (30 DAY)	
	STRAIN	NUMBER CONTROL	NUMBER TREATED				CONTROL	TREATED
10- 3-49	MG	24	24	450	10,000	5,000	54%	26%
10-10-49	MG	21	24	450	10,000	5,000	61	16
12- 5-49	MG	20	40	450	10,000	5,000	85	70
12-19-49	MG	48	48	450	10,000	5,000	81	41
Total		113	136				72%	41%
11- 7-49	MG	24	23	450	10,000	6,000	87%	39%
11-14-49	MG	24	48	450	10,000	6,000	95	44
11-21-49	MG	43	46	450	10,000	6,000	76	65
11-28-49	MG	24	48	450	10,000	6,000	83	54
Total		115	165				84%	52%

*Dose of drugs injected in 0.5 ml. saline subcutaneously once a day from the fourth through the twenty-eighth day postirradiation.

The abdomen and chest were opened aseptically and blood aspirated from the heart with a capillary pipette and cultured by streaking a drop on the surfaces of blood agar and eosin-methylene-blue plates and inoculating the remainder into 5 ml. Difco brain-heart infusion broth. The spleen was removed, cut in several places, and dropped into Difco brain-heart infusion broth. These cultures were incubated for twenty-four hours, and those showing growth were streaked onto blood agar and EMB agar. Negative cultures were incubated for an additional forty-eight hours and then streaked onto blood agar and EMB plates. Since so few anaerobes were found in blood cultures from killed mice,[1, 2] anaerobic cultures were not made of blood from dead animals.

RESULTS

Streptomycin.—Of the antibiotics tested streptomycin was the most effective in reducing the mortality of mice irradiated with the doses of x-ray used.

Table I summarizes the results of twenty-four experiments with streptomycin. It will be seen that doses of 1,000 or 2,000 μg a day were ineffective, but in each of the twenty experiments in which the daily dose of streptomycin ranged from 3,000 to 7,000 μg there was a significant reduction in mortality among the treated mice as compared with their controls. The optimum dose appeared to be in the neighborhood of 5,000 to 6,000 μg per day. The combined mortality curves of the two experiments begun May 9, 1949, are shown in Fig. 1.

The most striking therapeutic effect is to be seen in the last of the 6,000 μg experiments in which 16 per cent of the treated animals died and 92 per cent of the controls.

No autopsy cultures were made in the streptomycin series.

Streptomycin and Penicillin.—As shown in Table II, the addition of penicillin to streptomycin failed to improve the effectiveness of the latter.

Chloramphenicol.—The results are presented in Table III, in which experiments are grouped according to time and duration of medication. Within each group the experiments are listed in chronological order. In this and the follow-

ing tables mention is made of the occurrence in any experiments of infection with *Pseudomonas aeruginosa,* Proteus of all four species, or Salmonellae as demonstrated by autopsy cultures. The first four experiments which contained a total of seventy-four treated mice showed a striking therapeutic effect from chloramphenicol alone, 2 mg. per gram of food. In these four experiments, only 20 per cent of the treated mice died as compared with 62 per cent of the controls. The result with 5 mg. of chloramphenicol per gram of food was unsatisfactory, probably because the dose was too high.

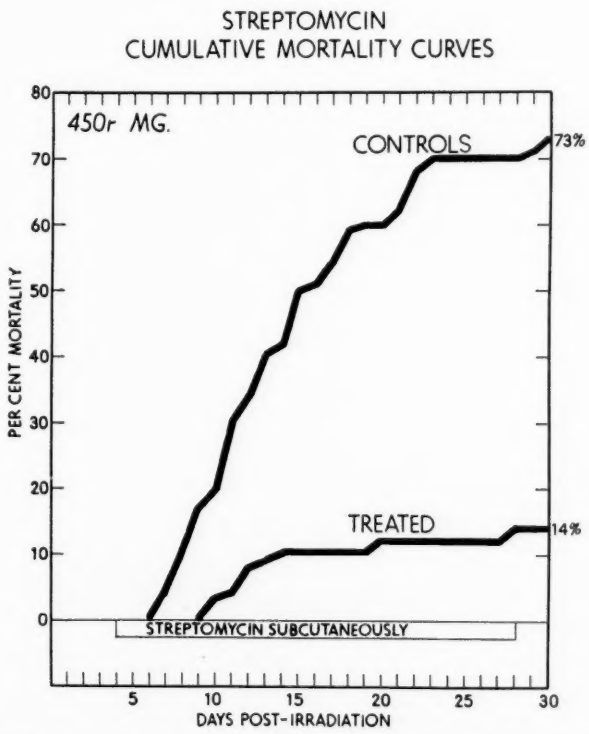


Fig. 1.—Effect on mortality of streptomycin, 6,000 micrograms subcutaneously once a day, for period indicated. Sixty-four treated and sixty-four control mice. 450 r = dose of x-ray. MG = Maple Grove mice, all males.

In the rest of the experiments the oral administration of chloramphenicol was supplemented by subcutaneous injections of streptomycin during the periods indicated in the tables. The results show a significant reduction in mortality among the treated mice except in those experiments in which *Pseudomonas aeruginosa* and Proteus infections were prevalent. Even in three of these experiments there was considerable evidence of protection. It will be noted that this epidemic of Pseudomonas infection occurred during the spring and summer of 1950. The over-all mortality during this period averaged 61 per cent for the mice treated with chloramphenicol and streptomycin, as compared with 88 per cent of the controls as shown in Table IV, in which the results of the experiments

TABLE III. RESULTS OF TREATMENT WITH CHLORAMPHENICOL BY MOUTH WITH OR WITHOUT STREPTOMYCIN SUBCUTANEOUSLY

DATE IRRADIATED	STRAIN	MICE NUMBER CONTROLS	MICE NUMBER TREATED	X-RAY DOSE R	CHLORO.-2 MG. DAYS GIVEN†	STREPTOMYCIN 6,000 μG DAYS GIVEN†	PREVALENCE OF INFECTION WITH PATHOGENS AS INDICATED BY AUTOPSY CULTURES	MORTALITY (30 DAY) CONTROL	MORTALITY (30 DAY) TREATED
5-14-51	RAP	34	35	550	4-28*		No autopsies	88%*	77%*
11-21-49	MG	21	8	450	4-27		No autopsies	85	12
11-28-49	MG	16	16	450	4-28		No autopsies	42	12
12- 5-49	MG	20	20	450	4-28		No autopsies	60	25
5- 7-51	RAP	31	30	550	4-20		No autopsies	58	23
Total— 2 mg.		88	74					62%	20%
2-20-50	MG	32	32	450	4-27	4-14	No autopsies	90%	27%
2-27-50	MG	30	30	450	4-11	12-17	No autopsies	70	17
4- 3-50	CF#1	36	36	600	4-28	9-14	Pseudo. and Proteus in many	83	58
5- 1-50	CF#1	30	29	550	4-14	15-20	Pseudo. in very many	100	93
6- 5-50	CF#1	31	31	500	4-28	4-16	Pseudo. and Proteus in many	100	64
6- 5-50	CF#1	32	31	500	4-28	8-14	Pseudo. and Proteus in many	96	58
8-28-50	CF#1	40	39	550	5-28	9-17	Sal. in many controls	87	12
9-11-50	CF#1	21	21	550	5-28	9-15	No autopsies	57	35
9-18-50	CF#1	39	39	550	5-28	9-15	Sal. in few controls	46	7
9-25-50	CF#1	43	43	550	4-22	9-15	Sal. and Proteus in few	44	18
7-24-50	CF#1	19	40	550	5-28	1-4 9-15	Proteus in many controls	73	7
8-21-50	CF#1	24	24	550	5-28	1-4 9-15	Sal. and Proteus in few controls	91	12
9-11-50	CF#1	21	20	550	5-28	1-4 9-15	Pseudo. in few controls	47	20
9-18-50	CF#1	38	39	550	5-28	1-4 9-15	Proteus in many	60	23
9-25-50	CF#1	43	22	550	5-23	1-4 9-15	Sal. and Proteus in few	44	18
7-20-50	CF#1	57	60	550	10-19	4-9	Proteus in many	40	25
8-14-50	CF#1	39	40	550	10-20	4-9	No autopsies	66	35
Total		575	576					60%	27%

*One experiment with 5 mg. per gram food.
†Days postirradiation.
RAP = Rockland Farms all-purpose mice, females.
Pseudo. = Pseudomonas aeruginosa; Sal. = Salmonella typhimurium.
Proteus = Proteus vulgaris, mirabilis, rettgeri, or morganii.

TABLE IV. THE EFFECT OF PSEUDOMONAS INFECTION ON THERAPEUTIC RESULTS

THERAPY	NUMBER OF MICE CONTROL	NUMBER OF MICE TREATED	MORTALITY (30 DAY) CONTROL	MORTALITY (30 DAY) TREATED
	Low incidence of Pseudomonas infection			
Chloramphenicol and streptomycin	425	429	42%	19%
Terramycin and streptomycin	255	243	65	33
	High incidence of Pseudomonas infection			
Chloramphenicol and streptomycin	150	147	88%	61%
Terramycin and streptomycin	302	294	96	83

are summarized according to the prevalence of such infection. The mortality for all of the experiments performed *in the absence* of serious Pseudomonas infection was 19 per cent for the treated mice and 42 per cent for the controls.

Fig. 2 presents the cumulative mortality curves in an illustrative experiment with chloramphenicol and streptomycin. The bacteria recovered from the heart's blood at autopsy are indicated alongside the rise in the mortality curve caused by the death of that mouse. In this experiment autopsy cultures were made on five mice. Three of them showed the presence of Salmonella in the heart's blood, one of them Proteus and one, Pseudomonas. Blood cultures on the one treated mouse which was autopsied showed no growth.

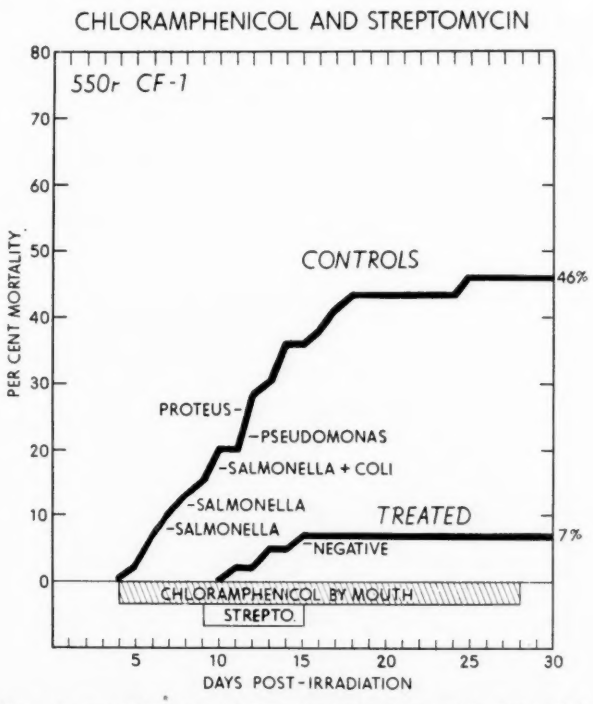


Fig. 2.—Effect on mortality of chloramphenicol mixed with food (2.0 mg. per gram of ground food) and streptomycin, 6,000 micrograms subcutaneously once a day, for periods indicated. Experiment dated Sept. 18, 1950, in Table III. 550 r = dose of x-ray. Thirty-nine treated and thirty-nine control mice. CF-1 = Carworth CF#1 mice, all females. Proteus, Pseudomonas, etc = bacteria recovered from heart's blood at autopsy. Negative = no growth.

Fig. 3 presents the results of a similar experiment performed at a time when drug-resistant strains of Pseudomonas and Proteus had become prevalent in the mouse room. The effect on the therapeutic response is shown in Table IV. The development of drug resistance by Pseudomonas will be discussed below.

Aureomycin.—As shown in Table V the most effective dose of aureomycin given alone was 7.0 mg. per gram of food. This dose of aureomycin was given to all of the mice which received additional treatment with streptomycin. The average mortality in all of the experiments with this combination of drugs was

23 per cent, compared with 47 per cent for their controls. The mortality curves of an illustrative experiment are shown in Fig. 4.

Terramycin.—As shown in Table V, terramycin was almost as effective as chloramphenicol and aureomycin in the absence of Pseudomonas infection. The mortality curves of an illustrative experiment are shown in Fig. 5. Six of the experiments with terramycin were carried out during an epidemic of drug-insensitive Pseudomonas infection (Fig. 3). Table IV shows the high death rate which resulted in spite of treatment with terramycin.

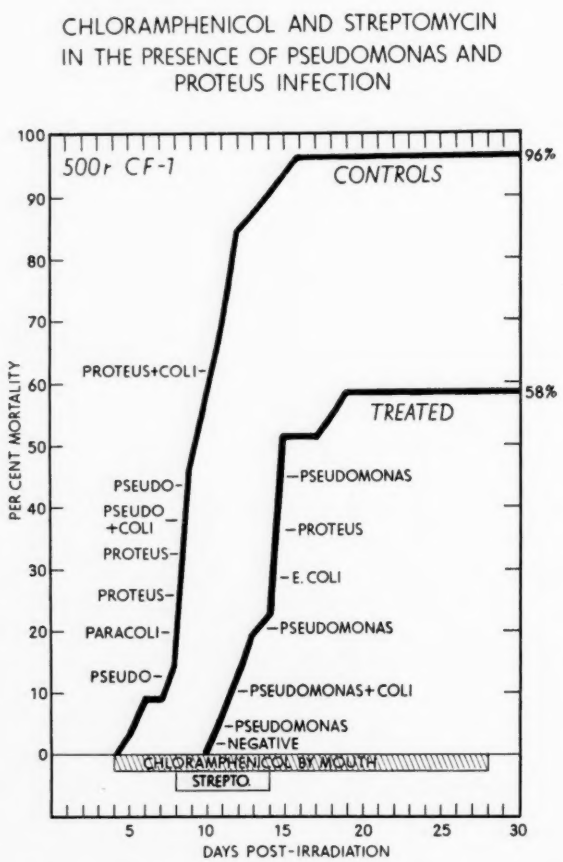


Fig. 3.—Experiment dated June 5, 1950, in Table III. Thirty-one treated and thirty-two control mice. See Fig. 2 for explanatory information.

Neomycin.—Mice receiving 1.45 mg. of neomycin subcutaneously once a day had a mortality of 74 per cent, as compared with 88 per cent for their controls. In another experiment animals were given 1.45 mg. of neomycin per day in the food and supplemented by subcutaneous injections of streptomycin during the first four days and the ninth to the fifteenth day had a mortality of 32 per cent, compared with 73 per cent for their controls.

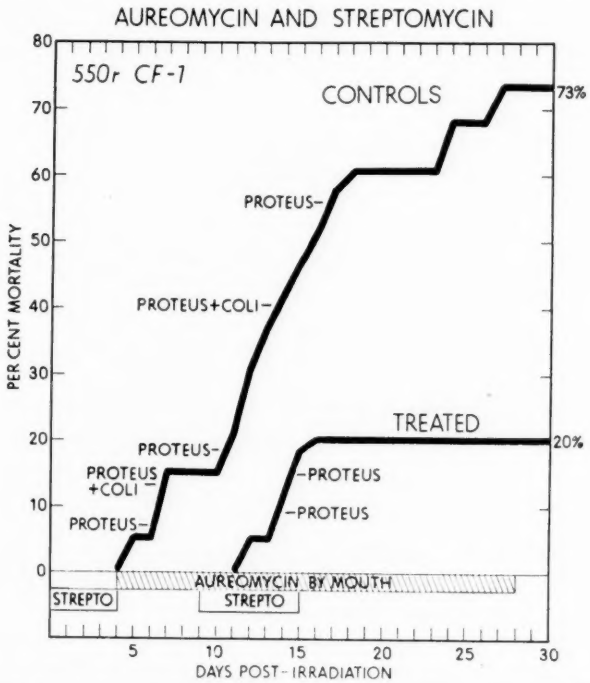


Fig. 4.—Aureomycin mixed with food (7.0 mg. per gram of ground food) and streptomycin, 6,000 micrograms subcutaneously once a day, for periods indicated. Experiment dated July 24, 1950, in Table V. Thirty-nine treated and nineteen control mice.

TABLE V. RESULTS OF TREATMENT WITH AUREOMYCIN BY MOUTH WITH OR WITHOUT STREPTOMYCIN SUBCUTANEOUSLY

DATE IRRADIATED	STRAIN	MICE NUMBER CONTROL	MICE NUMBER TREATED	X-RAY DOSE R	AUREOMYCIN AMT. MG.	AUREOMYCIN DAYS GIVEN*	STREPTOMYCIN 6,000 μG DAYS GIVEN*	PREVALENCE OF INFECTION WITH PATHOGENS AS INDICATED BY AUTOPSY CULTURES	MORTALITY (30 DAY) CONTROL	MORTALITY (30 DAY) TREATED
11-28-49	MG	16	16	450	2.5	4-28		No autopsies	42%	37%
12-12-49	MG	36	36	450	5.0	4-28		No autopsies	86	47
2-20-50	MG	31	31	450	7.0	4-28		No autopsies	61	26
5-14-51	RAP	34	36	550	2.0	4-28		No autopsies	88	47
			35		5.0	4-28				48
Total		117	134		2.0-7.0				74%	42%
7-20-50	CF#1	57	59	550	7.0	9-19	4-8	No autopsies	40%	15%
8-14-50	CF#1	66	29	550	7.0	9-20	4-8	No autopsies	66	29
7-24-50	CF#1	19	39	550	7.0	5-28	1-4 9-15	Proteus in many	73	20
9-11-50	CF#1	21	21	550	7.0	5-28	1-4 9-15	Pseudo. and Proteus in few	47	45
9-18-50	CF#1	38	19	550	7.0	5-28	1-4 9-15	Salmonella in many	66	15
9-25-50	CF#1	43	43	550	7.0	5-23	1-4 9-15	Sal. in many; Pseudo. in few	44	18
Total		244	210						47%	23%

*Days postirradiation.

TABLE VI. RESULTS OF TREATMENT WITH TERRAMYCIN BY MOUTH WITH OR WITHOUT STREPTOMYCIN SUBCUTANEOUSLY

DATE IRRADIATED	MICE			X-RAY DOSE R	TERRAMYCIN		STREPTOMYCIN DAYS GIVEN‡	PREVALENCE OF INFECTION WITH PATHOGENS AS INDICATED BY AUTOPSY CULTURES	MORTALITY (30 DAY)	
	STRAIN	NUMBER			AMT. MG.	DAYS GIVEN‡			CONTROL	TREATED
		CONTROL	TREATED							
1-23-50	MG	48	48	450	5.0	4-28		No autopsies	64%	87%
			48		7.0	4-28				58
2-20-50	MG	31	31	450	10.0	4-28		No autopsies	61	32
5-21-51	RAP	36	35	550	2.0	4-28		No autopsies	94	51
			35		5.0	4-28				40
6-11-51	RAP	36	35	550	7.0	4-28		No autopsies	80	31
Total		151	232						81%	49%
2-20-50	MG	32	16	450	7.0	4-28	4-16	No autopsies	90%	25%
7-24-50	CF#1	19	38	550	7.0	4-28	1-4	Proteus in many	73	26
							9-15			
9-18-50	CF#1	38	19	550	7.0	4-28	1-4	Proteus and Sal. in many	60	26
							9-15			
9-25-50	CF#1	43	44	550	7.0	4-23	1-4	Salmonella in many	44	20
							9-15			
6- 5-50	CF#1	31	32	500	7.0	4-28	1-16	Proteus and Pseudo. in many	100	50
6- 5-50	CF#1	32	30	500	7.0	4-28	8-14	Proteus in many; Pseudo. in few	97	33
5-22-50	CF#1	34	32	550	7.0	1-28	10-11	Pseudo. in very many	100	97
6-19-50	CF#1	29	31	500	7.0	9-20	4-8	Proteus in many	69	25
6-19-50	CF#1	29	31	500	7.0	9-20	4-8*	Proteus in many	69	38
7- 6-50	CF#1	41	40	550	7.0	7-17	1-6	Proteus in very many	78	70
5- 1-50	CF#1	30	30	550	7.0	4-14	15-20	Pseudo. in very many; Proteus in few	100	100
5-15-50	CF#1	34	32	550	7.0	4-12		Pseudo. in very many	100	100
5-15-50	CF#1	33	32	550	7.0	4-28	9-14	Pseudo. in very many	100	100
5-15-50	CF#1	35	35	550	7.0	4-28		Pseudo. in very many	100	100
5-22-50	CF#1	33	31	550	7.0	1-28	†	Pseudo. in very many	100	100
6-12-50	CF#1	40	40	500	7.0	4-28	4-28*	Pseudo. in many	72	32
6-12-50	CF#1	24	24	500	7.0	4-28	4-28*	Proteus in many	37	20
Total		557	537						80%	59%

*Oral streptomycin—10 mg. per gram food.
†Polymyxin (4-28)—0.5 mg per gram food.
‡Days postirradiation.

TABLE VII. RESULTS OF TREATMENT WITH THREE OR MORE DRUGS

DATE IRRADIATED	MICE			X-RAY DOSE R	CHLORAMPHENICOL 2.0 MG. DAYS GIVEN*	TERRAMYCIN 7.0 MG. DAYS GIVEN*	AUREOMYCIN 7.0 MG. DAYS GIVEN*	STREPTOMYCIN		"COMBIOTIC"		PREVALENCE OF INFECTION WITH PATHOGENS AS INDICATED BY AUTOPSY CULTURES	MORTALITY (30 DAY)	
	STRAIN	NUMBER CONTROL	NUMBER TREATED					DAYS GIVEN*	DOSE	DAYS GIVEN*	DOSE		CONTROL	TREATED
5/ 8/50	CF #1	35	35	550		13-18	4-8	9-12	6,000 μg			Pseudo. in many	100%	91%
5/ 8/50	CF #1	35	35	550	4-8		13-18	9-12	6,000 μg			Pseudo. in many	100	94
5/ 8/50	CF #1	35	35	550	13-18	4-8		9-12	6,000 μg			Pseudo. in many	100	100
5/22/50	CF #1	28	33	550		1-4		10-11	6,000 μg	4-28	2.0†	Pseudo. in very many	100	87
7/ 6/50	CF #1	40	40	550		2-28		7-12	6,000 μg	2-28	2.0†	Proteus in very many	90	75
8/ 7/50	CF #1	31	31	550				9-14	6,000 μg	4-8	2.0‡	Pseudo. and Proteus in few	90	26
8/ 7/50	CF #1	31	23	550				9-14	6,000 μg	15-24 4-8 15-24	2.0†	Pseudo. and Proteus in few	90	35
4/24/50	CF #1	32	32	550	4-28	4-28	4-28	4-28	10.0 mg.			Pseudo. in very many	84	75
5/ 1/50	CF #1	28	30	550	4-24	4-24	4-24	4-24	10.0 mg.			Pseudo. in very many	100	96
6/12/50	CF #1	24	23	500		4-28	4-28	4-28	10.0 mg.			Proteus in many	37	17

*Days postirradiation.
†2.0 Gm. per gram food (streptomycin sulfate 250.0 mg. per gram, polymyxin B 20.0 mg. per gram, bacitracin 5,000 units).
‡2.0 Gm. per gram food (streptomycin sulfate 250.0 mg. per gram, polymyxin B 20.0 mg. per gram, terramycin 100.0 mg. per gram).

UNIVERSITY OF MICHIGAN LIBRARIES

Polymyxin.—Polymyxin given subcutaneously in doses of 0.1 and 0.2 mg. in saline once a day for twenty-four days proved to be toxic and increased the mortality rate. Smaller doses of 0.02 and 0.05 mg. reduced it only slightly. Polymyxin alone was not administered by mouth but was used in combinations with other antibiotics in some of the experiments described below.

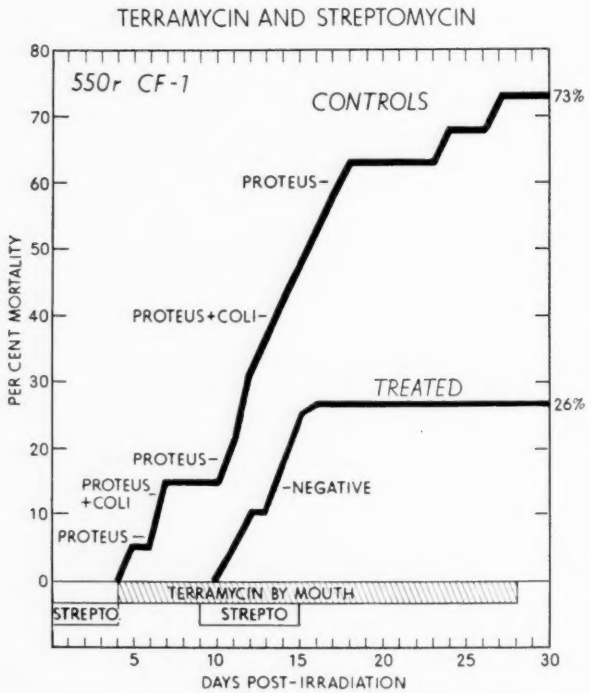


Fig. 5.—Terramycin mixed with food (7.0 mg. per gram of ground food) and streptomycin, 6,000 micrograms subcutaneously once a day, for periods indicated. Experiment dated July 24, 1950, in Table VI. Thirty-eight treated and nineteen control mice.

Several Antibiotics in Combination.—Table VII shows the results of the administration of three or more antibiotics. Unfortunately, six of these experiments were made during a period of serious Pseudomonas infection among the mice. Those which were carried out in the absence of such infection showed a marked therapeutic effect, but no greater than that obtained from using one of the orally administered drugs supplemented by subcutaneous streptomycin.

STATISTICAL ANALYSIS OF RESULTS*

The results reported above have been confirmed by statistical analysis. The differences between thirty-day survivals of control and antibiotic-treated

*For this analysis and for their helpful cooperation the authors are indebted to George A. Sacher, Sylvanus A. Tyler, and Joan M. Gurian of the Division of Biological and Medical Research, Argonne National Laboratory. Their methods will be described and discussed in detail elsewhere.

cages were tested by Chi-square. In twenty-five of forty-one comparisons, the difference was significant at the 5 per cent level. In every case where the difference was significant, the antibiotic-treated cage showed the greater survival. When all experiments which received similar antibiotic treatments were pooled and the control-therapy differences tested for these pooled groups (Tables I, II, III, V, VI, and VII), the differences were found to be significant in every case. There is therefore no question about the significance of the protection afforded by the antibiotic treatments.

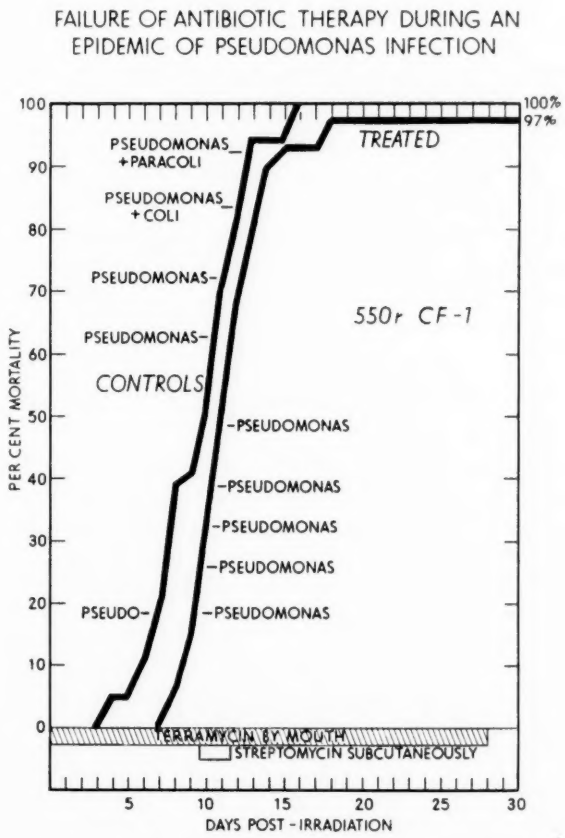


Fig. 6.—Treatment same as for Fig. 5. Experiment dated May 22, 1950, in Table VI. Thirty-two treated and thirty-four control mice.

An estimate was also made of the relative therapeutic effectiveness of the different therapeutic schedules. It was not possible to compare directly the differences in percentages of thirty-day survivors in control and treated groups, but it was shown that the mean difference of probits of control and treated cages is a reasonably good measure of the effectiveness of an antibiotic pattern. By this criterion it could be shown that there were no highly significant differences in therapeutic effectiveness between the various patterns of antibiotic therapy tested.

DISCUSSION

These experiments demonstrate that the mortality of mice exposed to moderate doses of x-radiation can be reduced by the administration of those antibiotics which are active against members of the ordinary intestinal flora. This result was anticipated by the earlier observation[1, 2] that severe bacteremia, amounting to an overwhelming sepsis, occurred in a large proportion of mice during the second and third weeks after irradiation—the period of greatest mortality. The protective action of antibiotics supports the conclusion drawn from that observation, viz., that infection contributes an important factor to the death of such mice.

Streptomycin was the most effective of the antibiotics used. The addition of penicillin to streptomycin failed to improve the therapeutic effect of the latter. The next most beneficial drugs were chloramphenicol, aureomycin, and terramycin. In most experiments treatment with these three drugs was supplemented by subcutaneous injections of streptomycin during the second week postirradiation, the period when bacteremia most often occurred.

Combinations of three or more antibiotics were no better than those mentioned above.

The superiority of streptomycin to the other antibiotics was anticipated. There is abundant evidence in the literature that it is the most active against the various bacterial species responsible for the fatal septicemias observed in irradiated mice.

The effectiveness of streptomycin seemed to persist throughout the course of treatment, which in most experiments lasted twenty-four days. This observation, if verified by additional experiments, will be surprising, for bacterial resistance to streptomycin develops very rapidly. The point is under investigation.

It is quite possible that the last week of treatment was superfluous in most experiments, because the greatest number of deaths from infection occurred during the second and third weeks postirradiation. In some of the early experiments an occasional mouse died later and it seemed advisable to prolong treatment through the fourth week. This schedule, once begun, was continued for the sake of uniformity.

The great variation in mortality among the untreated, control mice in different experiments has been a disquieting occurrence, as it seems to have been for other workers who have subjected many groups of animals to carefully standardized doses of x-ray.

These fluctuations in mortality were largely, but not entirely, explained by the blood cultures made on autopsied mice during the second year of the study. For the results showed that all instances of unusually high mortality were associated with a high incidence of infection with Pseudomonas, Proteus, or Salmonella. There were, however, a few experiments in which the mortality was relatively low, in spite of the demonstrated presence of Proteus infection in several mice.

Our data on the prevalence of infection with these pathogens are admittedly incomplete, because they derived from cultures on such mice as could be autopsied immediately after death. In most experiments, nevertheless, a sufficient number of bacteriologic observations were made to indicate whether or not infection with any of these microorganisms had assumed epidemic proportions.

The autopsy cultures also explained many of the therapeutic failures. Whenever drug-resistant strains of Pseudomonas became established in a group of mice, the mortality always approached 100 per cent. Epidemics of Proteus and Salmonella also increased the mortality, but not as seriously as Pseudomonas. These epidemics of Pseudomonas infection should have been anticipated because many strains of Pseudomonas are naturally resistant to therapeutic doses of chloramphenicol, aureomycin, and terramycin,[14, 15] and acquired resistance to streptomycin is rapidly developed.

On four occasions the effectiveness of antibiotic therapy was seriously reduced by such epidemics in the same mouse room. It then became necessary to terminate all existing experiments and to sterilize all equipment. Such a house cleaning was always followed by a series of experiments in which antibiotic therapy was once again effective. These distressing episodes served to emphasize the importance of infection as a factor in the death of mice subjected to moderate doses of ionizing radiation.

Our efforts to control the spread of infection with drug-resistant strains of pathogenic bacteria were unsuccessful until a routine was established which protected each cage from contamination as far as was practicable in a rather crowded mouse room. The most rewarding improvement in technique was the change from chemical to heat sterilization of water bottles and drinking tubes.

It should be pointed out that we were never able to reduce the mortality to zero even with streptomycin. It is possible that more effective antibiotic therapy might have lowered the mortality of treated mice still further. It seems more likely, however, that a certain proportion of the animals had suffered irreparable damage from irradiation and could not be kept alive by preventing the development of generalized infection. This supposition is supported by the fact that some of the treated mice which died had negative blood cultures. Examples are shown in Figs. 2, 3, and 5.

None of the antibiotics used was ideal for the purposes at hand. Streptomycin, the most effective one, is somewhat toxic in the doses used, and is a drug to which bacterial resistance develops rapidly. Chloramphenicol, aureomycin, and terramycin are incapable of controlling infection with most strains of pseudomonas and many of Proteus, both of which were present in the breeds of mice used.

The data here presented emphasize the importance of making repeated experiments using large numbers of animals to evaluate the effectiveness of therapeutic agents.

Although the results of these experiments demonstrated that the administration of appropriate antibiotics significantly reduced the mortality of irradi-

ated mice, it must not be assumed that similar treatment would produce a similar result in man. In irradiated mice the bacteria of the intestinal flora are apparently the only ones which give rise to generalized infection. In man, on the other hand, there are not only the intestinal bacteria, but those of the respiratory tract and skin which are potential causes of infection following exposure to ionizing radiation.

SUMMARY

Young adult mice of three different strains were subjected to total body x-radiation in a single exposure. The dose of x-ray varied from 450 to 600 r.

The mice were treated with a variety of antibiotics alone or in combination. Each experiment included a group of untreated control mice which had been irradiated at the same time with the same dose of x-ray. The number of controls usually equalled or approximated the number of treated mice.

The administration of streptomycin resulted in the greatest reduction in mortality. It was given once a day by subcutaneous injection. The addition of penicillin to streptomycin failed to improve the effectiveness of the latter. Chloramphenicol, aureomycin, and terramycin given by mouth (mixed in food) all caused significant reductions in mortality. When the oral treatment with these drugs was supplemented by daily injections of streptomycin during the second and third weeks postirradiation, the degree of protection was increased. This procedure was as effective as combinations of three or more antibiotics.

Neomycin was ineffective. Polymyxin proved to be too toxic.

Infections with Pseudomonas, Proteus, or Salmonella were not well controlled by treatment with chloramphenicol, aureomycin, or terramycin. When such infections became prevalent among the mice the mortality increased in both treated and control groups. Epidemics caused by drug-resistant strains of these bacteria could not be controlled. These observations emphasize the importance of infection as a lethal factor in mice exposed to moderate doses of x-radiation.

REFERENCES

1. Miller, C. P., Hammond, C. W., and Tompkins, M.: The Incidence of Bacteremia in Mice Subjected to Total Body X-Radiation, Science **111**: 540-41, 1950.
2. Miller, C. P., Hammond, C. W., and Tompkins, M.: Role of Infection in Radiation Injury, J. LAB. & CLIN. MED. **38**: 331, 1951.
3. Miller, C. P., Hammond, C. W., and Tompkins, M.: Reduction of Mortality From X-Radiation by Treatment With Antibiotics, Science **111**: 719-20, 1950.
4. Miller, C. P., and Hammond, C. W.: The Role of Infection in Radiation Injury, Trans. Assn. Am. Phys. **63**: 155-60, 1950.
5. Miller, C. P., and Hammond, C. W.: The Effect of Terramycin on Post-Irradiation Infection in Mice, Ann. N. Y. Acad. Sc. **53**: 303-8, 1950.
6. Howland, J. W., Furth, F. W., and Coulter, M.: The Effect of Aureomycin and Antibiotics on Whole Body Irradiation. Unclassified Report 94, University of Rochester, Atomic Energy Project dated 10-14-49.
7. Furth, F. W., Coulter, M., and Howland, J. W.: The Effect of Crude and Crystallized Aureomycin on the Weight of Normal and X-Radiated Rats, Unclassified Report 152, University of Rochester, Atomic Energy Project, dated 2-1-51, pp. 39-41.
8. Furth, F. W., and Coulter, M.: The Effect of Aureomycin on the Radiation Syndrome on Dogs, Unclassified Report 116, University of Rochester, Atomic Energy Project, dated 4-27-50.

9. Allen, J. G., Moulder, P. V., and Enerson, V. M.: Pathogenesis and Treatment of the Post-Irradiation Syndrome, J. A. M. A. **145**: 704-11, 1951.
10. Cronkite, E. P.: The Clinical Manifestations of Acute Irradiation Illness in Goats, U. S. Naval Medical Bulletin **49**: 199-215, 1949.
11. Cronkite, E. P., and Jackson, D. P.: Personal communication to the authors.
12. Koletsky, S., and Christie, J. H.: Effect of Antibiotics on Mortality From Internal Radiation, Proc. Soc. Exper. Biol. & Med. **75**: 363-66, 1950.
13. Herrell, W. E., Heilmann, F. R., and Wellman, W. E.: Some Bacteriologic, Pharmacologic and Clinical Observations on Terramycin, Ann. N. Y. Acad. Sc. **53**: 448-58, 1950.
14. Alexander, H. E., Leidy, G., and Redman, W.: Comparison of the Action of Streptomycin, Polymyxin B, Aureomycin and Chloromycetin on H. Pertussis, H. Parapertussis, H. Influenzae and Five Enteric Strains of Gram-Negative Bacilli, J. Clin. Investigation **28**: 867-70, 1949.
15. English, A. R., and Gelwicks, P. C.: The In Vitro Microbial Resistance Pattern to Terramycin, Antibiotics and Chemotherapy **1**: 118-24, 1951.

LABORATORY METHODS

SIMPLE METHOD FOR DEMONSTRATING THE "L.E." PHENOMENON IN PERIPHERAL BLOOD

AUSTIN S. WEISBERGER, M.D., GORDON C. MEACHAM, M.D., AND ROBERT W. HEINLE, M.D.
CLEVELAND, OHIO

THE demonstration of leukocytic inclusion bodies ("L.E." cells) and rosettes in bone marrow[1-3] or peripheral blood[4-7] has been helpful in the diagnosis of acute disseminated lupus erythematosis. A simple procedure, representing modification of other methods, has been devised to demonstrate the "L.E." phenomenon in peripheral blood. By accelerating the erythrocyte sedimentation with fibrinogen,[8] excellent separation of leukocytes is accomplished.

METHOD

Ten milliliters of venous blood are drawn with a syringe containing 0.1 to 0.2 ml. of liquid heparin (sodium heparin, 10 mg. [equivalent to 1,000 units] per milliliter). The mixture is placed in an ordinary glass tube of suitable capacity.

2. A fibrinogen solution is prepared by dissolving 310 mg. of bovine fibrinogen (Fraction I, Armour) in 10 ml. of distilled water. The mixture is shaken and allowed to stand for five to fifteen minutes at room temperature to insure complete solution.

3. Approximately 1.25 ml. of the fibrinogen solution are added to each 10 ml. of heparinized venous blood and mixed by inversion.

4. Rapid sedimentation of erythrocytes occurs after a brief lag period and progresses so that the volume of sedimented erythrocytes approaches packed cell volume within one hour. The plasma may be removed during this period with a Wintrobe pipette and is placed in a serologic test tube. The leukocyte and thrombocyte concentrations in plasma collected in this manner are almost equivalent to those present in whole blood.

5. The plasma-leukocyte suspension is allowed to stand at 37° C. or at room temperature for an additional thirty minutes. It is then centrifuged at 1,000 RPM for five minutes, the supernatant plasma decanted, and cover slip films made from the packed cells which are removed with a Wintrobe pipette or Sahli type (20 cubic millimeters) hemoglobin pipette.

RESULTS

Films prepared by this technique are composed almost entirely of large numbers of leukocytes and platelets, so that the finding of "L.E." cells is greatly

From the Department of Medicine, Western Reserve University School of Medicine, and University Hospitals.
Received for publication, Oct. 15, 1951.

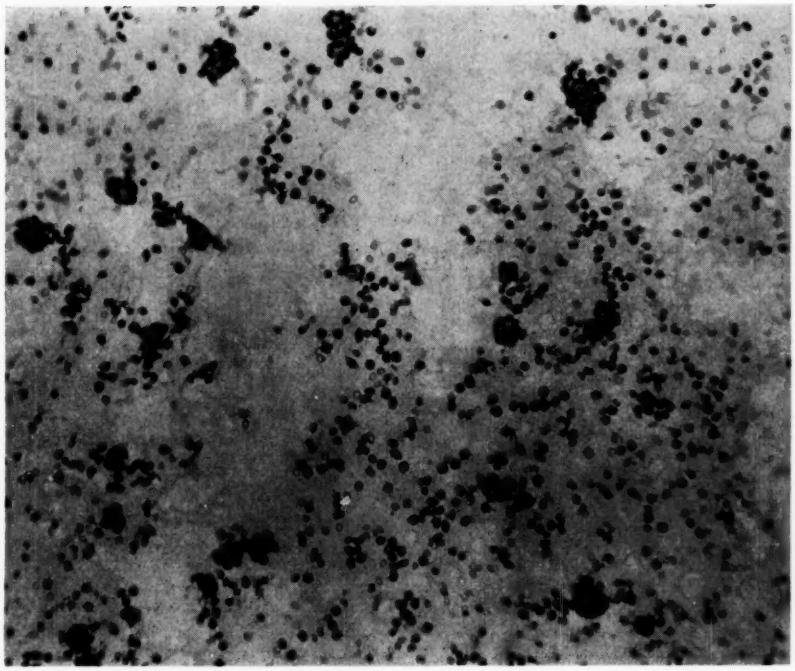


Fig. 1.—Rosette formation. (×144.)

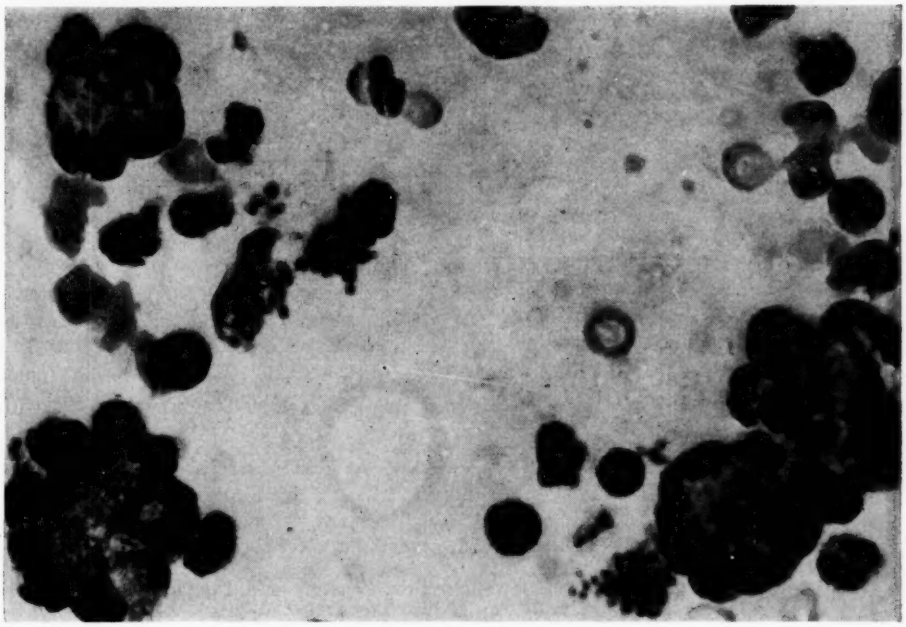


Fig. 2.—Rosette formation. (×966.)

facilitated even if the patient has a severe leukopenia. Only a small number of erythrocytes is present. Rosette formation was very striking in five patients with active acute disseminated lupus erythematosis (Figs. 1 and 2) and "L.E." cells were abundant (Fig. 3). Neither rosettes nor "L.E." cells were encountered in such preparations from ten normal subjects or from twenty-five patients with various diseases (chronic discoid lupus, polyarteritis nodosa, pemphigus, subacute bacterial endocarditis, hepatic cirrhosis, rheumatoid arthritis, amebic hepatitis, and fever of undetermined origin). The results with this method appear to be entirely comparable to those obtained when bone marrow content placed in plasma from the patient is used as a source of leukocytes.

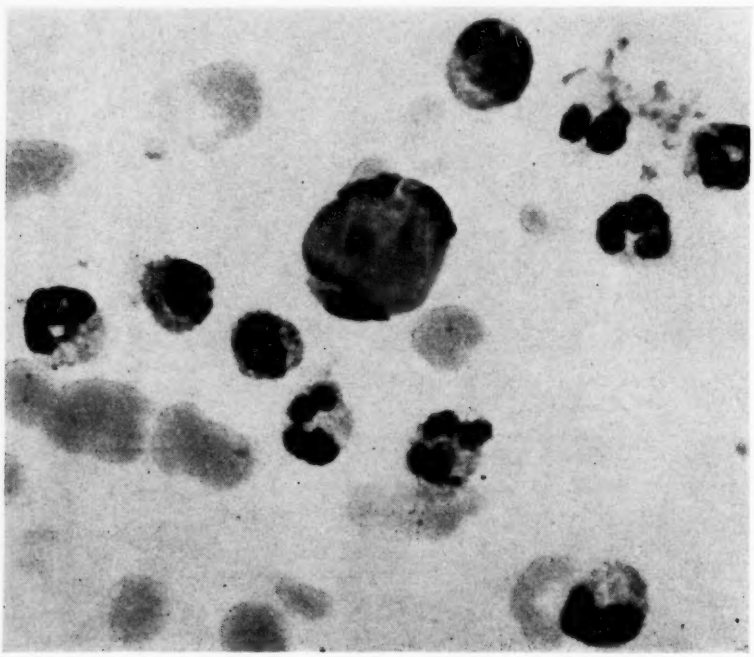


Fig. 3.—"L.E." cell. (×1300.)

SUMMARY

A simple method is described for the demonstration of "L.E." cells and rosettes in the peripheral blood of patients with disseminated lupus erythematosis. Use of fibrinogen allows rapid and nearly complete separation of leukocytes from erythrocytes.

REFERENCES

1. Hargraves, M. M., Richmond, Helen, and Morton, R.: Presentation of Two Bone Marrow Elements: the "Tart" Cell and the "L.E." Cell, Proc. Staff Meet., Mayo Clin. **23**: 25, 1948.
2. Haserick, J. R., and Sundberg, R. D.: The Bone Marrow as a Diagnostic Aid in Acute Disseminated Lupus Erythematosus, J. Invest. Dermat. **11**: 209, 1948.
3. Hargraves, M. M.: Production *In Vitro* of the "L.E." Cell Phenomenon: Use of Normal Bone Marrow Elements and Blood Plasma from Patients with Acute Disseminated Lupus Erythematosis, Proc. Staff Meet., Mayo Clin. **24**: 234, 1949.

4. Sundberg, R. D., and Lick, N. B.: "L.E." Cells in the Blood in Acute Disseminated Lupus Erythematosus, J. Invest. Dermat. **12**: 83, 1949.
5. Moffat, T. W., Barnes, S. S., and Weiss, R. S.: The Induction of the "L.E." Cell (Hargraves) in Normal Peripheral Blood, J. Invest. Dermat. **14**: 153, 1950.
6. Mathis, H. B.: A Simple Office Procedure for Demonstrating Lupus Erythematosus Cells in Peripheral Blood, Blood: 6: 470, 1951.
7. Suksta, A., and Conley, C. Lockard: Some Observations on the "L.E." Cell, J. LAB. & CLIN. MED. **37**: 597, 1951.
8. Minor, A. H., and Burnett, L.: A Method for Obtaining Living Leukocytes from Human Peripheral Blood by Acceleration of Erythrocyte Sedimentation, Blood **3**: 799, 1948.

SHEEP BLOOD AGAR FOR THE ISOLATION OF LANCEFIELD GROUPS OF BETA-HEMOLYTIC STREPTOCOCCI*

A. E. Feller, M.D.,** and David A. Stevens, A.B.
Cleveland, Ohio

INVESTIGATIONS of the role of beta-hemolytic streptococci in respiratory disease require reliable methods for characterizing streptococci isolated from throat swabs. Classification into groups and types by serological methods is essential for final identification. Previous experience in this laboratory has demonstrated that many strains of streptococci isolated from the oropharynx of either normal persons or patients with respiratory disease cannot be grouped even though the colonies show beta-hemolysis on horse blood agar. Thus, considerable time and materials are consumed in a fruitless attempt to identify such strains.

Recent studies have indicated that beta-hemolysis by surface or subsurface colonies on sheep blood agar is a simple and reliable indication that streptococci isolated from throat cultures may be grouped serologically, and also that sheep blood agar is as reliable as horse blood agar for the detection of group A streptococci but not for other groups of streptococci. The results of these studies are presented in this paper.

METHODS

Source of Cultures.—A population of approximately sixty families (approximately 250 individuals) was the source of the throat cultures. This population serves as a basis for a study of illness in families.[1] For one of the studies (Table II), throat swabs were also obtained from 300 well persons, chiefly medical students and professional personnel.

The throat was cultured by rubbing each tonsillar area and the oropharynx with a cotton swab. When duplicate cultures were required for certain of the comparative studies two swabs held in parallel were used simultaneously.

All swabs were transported to the laboratory in sterile test tubes and planted after a delay of not longer than two hours from the time the swabs were obtained. Under similar conditions comparative data[2] have shown that transportation of throat swabs placed in dry test tubes does not reduce the recovery rate of groups A, C, or G streptococci when compared with transportation of duplicate swabs in broth.

Technique of Isolating Streptococci.—All cultures were examined for beta-hemolytic streptococci using Pike's selective medium.[3, 4] The Pike technique has continued to be superior to the conventional streak plate method for detecting beta-hemolytic streptococci on throat swabs, especially when the organisms are present in small numbers.[5, 6] The swab was placed in blood broth containing sodium azide and crystal violet and incubated at 37° C. overnight. A loopful of culture was then streaked upon the surface and stabbed into the sub-

From the Departments of Preventive Medicine and of Medicine, School of Medicine, Western Reserve University, and the University Hospitals.

Received for publication, Oct. 17, 1951.

*This investigation was supported in part through the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, Office of The Surgeon General, U. S. Army, and by grants from the Brush Foundation, the Cleveland Foundation, the S. P. Fenn Trust, and Mr. Philip R. Mather.

**Present address: School of Microbiology, Department of Medicine, University of Virginia, Charlottesville, Va.

stance of a horse blood agar plate. The plate was incubated at 37° C. for twenty-four hours and examined with the unaided eye and with the aid of a widefield binocular microscope, since the latter has been shown to aid materially in the detection of colonies of streptococci.[7] To obtain pure cultures, subcultures were made to a second horse blood agar plate, incubated at 37° C. for twenty-four hours and then examined as above.

Organisms that exhibited beta-hemolysis around surface or deep colonies in horse blood agar and were gram-positive cocci in chains were tentatively considered to be beta-hemolytic streptococci. Confirmatory procedures included comparative studies of hemolysis on horse and sheep blood agar, grouping of the organisms serologically and, in certain instances, observations of the hemolytic properties on human and rabbit blood agar and on agar media containing erythrocytes only or various combinations of erythrocytes and serum.

Comparative Studies on Horse and Sheep Blood Agar.—The organisms in pure culture were streaked upon the surface and stabbed in to the substance of horse and sheep blood agar plates concurrently and the plates incubated at 37° C. for twenty-four hours. Without knowledge of the results of serologic classification, the resultant colonies were examined for hemolysis with the unaided eye and with the widefield colony microscope. The additional comparative studies of hemolysis on other media were made in the same manner.

Technique of Grouping.—The serological method of Lancefield[8] was used. Formamide extracts[9] were employed and, in addition, Maxted's "enzyme" technique[10] was used to prepare extracts of the organisms isolated in 1950 and subsequently. Experience with Maxted's technique confirms that reported by others.[10, 11] Sera for Lancefield groups A, B, C, D, E, F, G, H, and L were routinely employed.*

Media Employed.—Blood agar plates contained approximately 6 per cent defibrinated horse, sheep, rabbit or human blood. The agar base contained either beef heart infusion or Difco tryptose blood agar base at a pH of 7.5 before the blood was added. Comparative studies of these two media[2] have shown them to be of equal reliability for detecting beta-hemolytic streptococci from throat swabs. Colonies of such streptococci on blood agar media with the Difco base tend to be smaller but the zone of hemolysis is generally larger than on similar plates with beef heart infusion base.

Agar plates that contained only erythrocytes were made by centrifuging defibrinated blood, removing the serum, washing the cells once with physiologic saline solution, and employing sufficient sedimented cells to match the color of regular plates. Plates which contained erythrocytes from one species and serum from another species employed a mixture of approximately equal proportions of cells and serum in sufficient quantity to match the color of the regular plates.

RESULTS

Study of Organisms Producing Beta-Hemolysis on Horse Blood Agar.—Cultures from the population of 60 families were employed in this study, approximately half of the cultures being obtained in October, 1948, and the other half in April, 1950. A total of 125 strains of streptococci producing beta-hemolysis on horse blood was isolated from 566 cultures. The hemolytic properties of colonies of these 125 strains on horse and sheep blood agar were then determined and correlated with the results of attempts to group them serologically (Table I).

All of the 101 strains that were beta-hemolytic on sheep blood agar could be grouped. Twenty-four strains failed to produce beta-hemolysis either on the surface or within the substance of sheep blood agar and none could be grouped; of these 24 strains, 19 produced beta-hemolysis on the surface, and 22 produced beta-hemolysis within the substance of horse blood agar.

*The antisera used in this study were supplied in part by the laboratories of the New York State Department of Health and the Communicable Disease Center, U. S. Public Health Service.

TABLE I. LANCEFIELD GROUPS OF 125 CONSECUTIVE STRAINS OF BETA-HEMOLYTIC STREPTOCOCCI ACCORDING TO THE TYPE OF HEMOLYSIS ON HORSE BLOOD AGAR AND SHEEP BLOOD AGAR

HEMOLYSIS ON BLOOD AGAR				GROUP (LANCEFIELD)					NO GROUP*	TOTAL
HORSE		SHEEP								
STREAK	STAB	STREAK	STAB	A	B	C	F	G		
Beta	Beta	Beta	Beta	41	20	13	8	9		91
Beta	Beta	Alpha	Beta		1	7		1		9
Alpha	Beta	Alpha	Beta			1				1
Beta	Beta	Alpha	Alpha						17†	17
Alpha	Beta	Alpha	Alpha						5	5
Beta	Alpha	Alpha	Alpha						2	2
Total				41	21	21	8	10	24	125

*These strains could not be grouped with sera for groups, A, B, C, D, E, F, G, H, or L.
†One of these strains was positive with group H serum with a formamide extract, but was twice negative with the same serum with extracts prepared with Maxted's enzyme.

Ten of the groupable strains were beta-hemolytic within the substance of sheep blood agar but were alpha-hemolytic on the surface. Eight of these strains were group C, one was group B and one was group G. Colonies of group C strains, as a rule, exhibited a zone of hemolysis on sheep blood agar which was cleared of intact cells but was slightly or definitely green. Thus, classification of surface hemolysis of group C strains on sheep blood agar as alpha or beta was often a matter of personal opinion. The hemolytic zone about deep colonies of such strains was always clearly beta in type, however, and offered no difficulty of classification.

Although 20 strains of group B streptococci are tabulated as having exhibited beta-hemolysis on horse and sheep blood agar (Table I), only the deep colonies on these media produced unmistakable beta-hemolysis. Almost without exception surface colonies of group B streptococci on both horse and sheep blood agar produced a rather narrow zone of hemolysis which was colorless, but which contained recognizable cells. Thus, classification of hemolysis of surface colonies of group B strains as beta in type was an arbitrary decision between alpha prime and beta-hemolysis.

These data indicated that (1) strains of streptococci originally selected because they were beta-hemolytic on horse blood agar could be grouped serologically without exception when they were also beta-hemolytic on sheep blood agar, and (2) none of the strains could be grouped that were beta-hemolytic on horse blood agar but alpha-hemolytic on sheep blood agar. A study was therefore made to determine whether or not original isolations could be made on sheep blood agar rather than horse blood agar without failure to detect groupable strains.

Comparison of Sheep Blood Agar and Horse Blood Agar for the Isolation of Groupable Streptococci From Throat Swabs.—For this study, 472 consecutive throat cultures, using two swabs, were obtained from the members of the 60 families and 300 cultures were obtained in duplicate from medical students and professional personnel. One of each of the 772 pairs of swabs was incubated in Pike's broth containing horse blood and then plated on horse blood agar from which beta-hemolytic colonies were picked to sheep blood agar for serologic grouping. The other of each pair of swabs was incubated in Pike's broth con-

taining sheep blood and then plated on sheep blood agar from which beta-hemolytic colonies were picked to sheep blood agar for serologic grouping. In an attempt to minimize errors due to failure of recognition, the negative plate of a pair was always re-examined for beta-hemolytic colonies. In addition, when a groupable strain of a streptococcus was detected on horse blood agar and not on sheep blood agar, or vice versa, the strain was always subcultured to the other medium to investigate the possibility that the organism would not grow in that medium. No such failures of growth were encountered.

The results showed that sheep blood agar and horse blood agar were equally reliable for the detection of group A streptococci (Table II). Horse blood agar was more efficient than sheep blood for detecting groups B, C, F, and G streptococci. Although certain of the differences may have been due to unequal distribution of organisms between the two swabs, such differences would be expected to distribute at random.

TABLE II. COMPARISON OF HORSE BLOOD AGAR AND SHEEP BLOOD AGAR FOR THE ISOLATION OF GROUPABLE STREPTOCOCCI FROM 772 THROAT SWABS TAKEN IN DUPLICATE

	NUMBER OF STRAINS ISOLATED, BY LANCEFIELD GROUPS AND ACCORDING TO MEDIUM EMPLOYED			
GROUP	S+* H+	S0 H+	S+ H0	TOTAL
A	44	7	10	61
B	35	19	10	64
C	18	6	4	28
F	6	10	7	23
G	13	5	1	19
Total				195

*S = sheep blood agar.
H = horse blood agar.
+ = groupable streptococcus present.
0 = groupable streptococcus absent.

Reliability of Beta-Hemolysis on Sheep Blood Agar as an Indication That Strains of Streptococci Can Be Grouped Serologically.—On the basis of the data presented above the procedure in this laboratory has been to employ horse blood agar plates for the detection of beta-hemolytic strains of streptococci from throat swabs. Such strains are then subcultured to sheep blood agar and only organisms which are beta-hemolytic on the latter are selected for serologic grouping. Data have been analyzed to determine the reliability of the presence of beta-hemolysis on sheep blood agar as an indication that such strains can be grouped.

During the months of April, May, and June, 1949, and April and May, 1950, a total of 6,235 throat cultures was examined for beta-hemolytic streptococci. From these cultures, 1,812 strains of streptococci were obtained that appeared to be beta-hemolytic on horse blood agar, and were subcultured to sheep blood agar; 1,224 strains (67.5 per cent) were beta-hemolytic on sheep blood agar (surface or deep colonies), and 1,193 (97.4 per cent) of them could be grouped. Review of the thirty-one strains (2.6 per cent) which could not be grouped revealed that twenty-five grew as definite pin-point sized colonies. Such strains are usually group F and are, as a rule, difficult to group because they grow

poorly on subculture and satisfactory grouping extracts are not always obtained. Often two or three extracts must be prepared before successful grouping is accomplished. The other six strains were apparently typical beta-hemolytic colonies on sheep blood agar but could not be grouped. The remaining 588 strains (32.4 per cent) were not beta-hemolytic on sheep blood agar and grouping was not attempted since, as noted in Table I, the procedure was so unsuccessful with such strains. Thus, the routine use of sheep blood agar resulted in the elimination of approximately one-third of the strains from further study and the inclusion of strains 97.4 per cent of which could be grouped.

TABLE III. TYPE OF HEMOLYSIS PRODUCED BY SURFACE AND DEEP COLONIES ON HORSE, SHEEP, HUMAN, AND RABBIT BLOOD AGAR BY FIFTEEN STRAINS OF STREPTOCOCCI WHICH COULD NOT BE GROUPED (LANCEFIELD) EMPLOYING GROUPS A, B, C, D, E, F, G, H, AND L SERA

STRAIN NO.	TYPE OF BLOOD AND RESULTANT HEMOLYSIS OF SURFACE AND DEEP COLONIES							
	HORSE		SHEEP		HUMAN		RABBIT	
	STREAK	STAB	STREAK	STAB	STREAK	STAB	STREAK	STAB
1	β	β	α	α	α	α	?β*	?β
2, 4, 14, 15	β	β	α	α	β	β	β	β
3	α	β	α	α	β	β	β	β
5, 6, 7, 13	α	β	α	α	β	β	?β	β
8, 9	β	β	α	α	α	α	β	β
10, 11, 12	β	β	α	α	α	α	α	β

*?β = weak beta hemolysis.

Hemolysis on Horse, Sheep, Human and Rabbit Blood Agar by Fifteen Nongroupable Strains and Eleven Groupable Strains.—The observation that certain strains of streptococci produced beta-hemolysis on horse blood agar, yet were alpha-hemolytic on sheep blood agar and could not be grouped, suggested further study of their hemolytic behavior on the blood of other species. Accordingly, the hemolytic action of fifteen such nongroupable strains was compared with that of eleven groupable strains on agar plates containing human, rabbit, horse and sheep bloods, respectively. The plates were streaked and stabbed so as to give both surface and deep colonies. The nongroupable strains were isolated in April, 1950; the groupable strains were isolated currently.

All of the fifteen nongroupable strains showed alpha-hemolysis on sheep blood but produced beta-hemolysis on horse and rabbit bloods. More than half of them were beta-hemolytic on human blood (Table III). Although on horse blood the areas of beta-hemolysis around approximately half of the fifteen strains were not wide and clear, the other half were typically and clearly beta-hemolytic and would readily be so classified even by an inexperienced observer. Thus, sheep blood was the only one of the four bloods ordinarily available which served reliably to indicate the strains of streptococci which could not be grouped serologically.

Study of eleven current strains of groupable streptococci (six of group A, one of group B, one of group C, and three of group G) on these four media revealed that all strains produced typical beta-hemolysis (surface and deep colonies) on all four types of blood agar. Although these data are few, they suggest that human and rabbit blood agar might serve as well as horse blood agar for isolating groupable streptococci.

Hemolysis by Two Groupable and Two Nongroupable Strains on Media Prepared with Defibrinated Blood, Erythrocytes, and Combinations of Erythrocytes and Serum from Four Species.—These data (Table IV) show that alpha-hemolysis produced by the two nongroupable strains occurred only on those media which contained sheep erythrocytes. The observation that alpha-hemolysis by the nongroupable strains resulted when sheep erythrocytes were added to horse, human, or rabbit serum suggests that the factor which permits sheep blood agar to differentiate between groupable and nongroupable streptococci is associated with the erythrocytes rather than the serum.

TABLE IV. TYPE OF HEMOLYSIS PRODUCED BY TWO GROUPABLE AND TWO NONGROUPABLE STRAINS OF STREPTOCOCCI ON BLOOD AGAR PLATES PREPARED WITH DEFIBRINATED BLOOD, ERYTHROCYTES, AND COMBINATIONS OF ERYTHROCTYES AND SERUM FROM FOUR SPECIES

TYPE OF BLOOD AGAR PLATES EMPLOYED	STREPTOCOCCUS EMPLOYED AND TYPE HEMOLYSIS							
	GROUP A		GROUP C		NO GROUP		NO GROUP	
	STREAK	STAB	STREAK	STAB	STREAK	STAB	STREAK	STAB
Defibrinated blood:								
Horse	β	β	β	β	β	β	β	β
Sheep	β	β	β	β	α	α	α	α
Rabbit	β	β	β	β	β	β	β	β
Human	β	β	β	β	β	β	β	β
Erythrocytes:								
Horse	β	β	β	β	β	β	β	β
Sheep	β	β	β	β	α	α	α	α
Rabbit	β	β	β	β	β	β	β	β
Human	β	β	β	β	β	β	β	β
Horse cells + sheep serum	β	β	β	β	β	β	β	β
Sheep cells + horse serum	β	β	β	β	α	α	α	α
Rabbit cells + human serum	β	β	β	β	β	β	β	β
Human cells + rabbit serum	β	β	β	β	β	β	β	β
Sheep cells + rabbit serum	β	β	α	β	α	α	α	α
Sheep cells + human serum	β	β	β	β	α	α	α	α

DISCUSSION AND SUMMARY

The results of this study show that strains of streptococci isolated from throat cultures and found to be beta-hemolytic on sheep blood agar can almost invariably be grouped serologically (Lancefield). The chief exceptions are strains which grow on solid media as pin-point sized colonies, are difficult to grow in sufficient quantity to permit preparation of a potent extract for serologic grouping and, when successfully grouped, are usually found to be group F. An occasional strain is encountered (six of a total of 1,224), however, which grows as regular sized colonies, is beta-hemolytic on sheep blood and cannot be grouped serologically. These may represent technical failures or groups for which sera were not available.

For routine laboratory purposes, the results indicate that the use of sheep blood agar for the recognition of group A beta-hemolytic streptococci from

throat cuutures constitutes a reliable technique and one that will result in a considerable saving of time and materials. It has the additional advantage that *Hemophilus hemolyticus* either fails to grow or grows very poorly on sheep blood agar.[12] The precaution should be taken, however, to stab into the agar as well as to streak the surface, since approximately 10 per cent of strains may give beta-hemolysis only around deep colonies.

Although the data show that sheep blood agar and horse blood agar are of essentially equal reliability for the detection of group A streptococci, horse blood agar for subculture of Pike's broth appears to be somewhat better than sheep blood agar for the recognition of strains of groups B, C, F, and G streptococci. Rabbit blood agar and human blood agar were not evaluated for the isolation of streptococci.

The identity of the strains that were beta-hemolytic on horse blood agar and alpha-hemolytic on sheep blood agar and that could not be grouped serologically is unknown. It is possible that they belong to a group, or to groups, as yet unknown or for which sera were not available. Study of fifteen such strains showed that the majority of them were also beta-hemolytic on rabbit and human blood agar. On horse blood agar approximately half of these fifteen strains produced typical and obvious wide zones of beta-hemolysis. The others produced less obvious zones of beta-hemolysis but all were readily recognized as beta-hemolytic by three experienced observers.

In general, studies employing such cultural characteristics of streptococci as colonial morphology and hemolysis have not appeared to offer a reliable criterion of groupability.[2, 13-15] Observation of the zones of hemolysis about nongroupable and groupable strains, including group A, has always revealed strains in each category that produced beta-hemolysis which was less obvious than the usual wide clear zones.[2] The experience thus far with sheep blood indicates that hemolysis of the sheep erythrocyte provides a practical and sufficiently reliable criterion of groupability to warrant routine adoption of sheep blood media.

Comparison of the hemolytic behavior of four groupable and nongroupable strains on various combinations of erythrocytes and serum from four species indicate that susceptibility to beta-hemolysis is associated with the sheep erythrocytes and is unaffected by other homologous or heterologous serum.

CONCLUSION

The production of beta-hemolysis on sheep blood agar by strains of beta-hemolytic streptococci is a reliable indication that such strains can be grouped serologically.

The technical assistance of Mary K. Dixon, Bernice Miraldi, Nancy Cope, and Ruth Genutis is gratefully acknowledged.

REFERENCES

1. Dingle, J. H., Badger, G. F., Feller, A. E., Hodges, R. G., Jordan, W. S., Jr., and Rammelkamp, C. H., Jr.: A Study of Respiratory Infections in Families, Tr. A. Am. Physiol. 62: 99, 1949.
2. Unpublished data.

3. Pike, R. M.: An Enrichment Broth for Isolating Hemolytic Streptococci from Throat Swabs, Proc. Soc. Exper. Biol. & Med. **57**: 186, 1944.
4. Pike, R. M.: The Isolation of Hemolytic Streptococci from Throat Swabs, Experiments with Sodium Azide and Crystal Violet in Enrichment Broth, Am. J. Hyg. **41**: 211, 1945.
5. Pike, R. M., and Fashena, G. J.: Frequency of Hemolytic Streptococci in the Throats of Well Children in Dallas, Am. J. Pub. Health **36**: 611, 1946.
6. Commission on Acute Respiratory Diseases: The Single Throat Culture as an Index of the Bacterial Flora of the Respiratory Tract, Am. J. Hyg. **50**: 168, 1949.
7. Commission on Acute Respiratory Diseases, in collaboration with Mickle, W. A., and Oliver, T. J.: Problems in Determining the Bacterial Flora of the Pharynx, Proc. Soc. Exper. Biol. & Med. **69**: 45, 1948.
8. Lancefield, R. C.: A Serological Differentiation of Human and Other Groups of Hemolytic Streptococci, J. Exper. Med. **57**: 571, 1933.
9. Fuller, A. T.: Formamide Method for Extraction of Polysaccharides from Haemolytic Streptococci, Brit. J. Exper. Path. **19**: 130, 1938.
10. Maxted, W. R.: Preparation of Streptococcal Extracts for Lancefield Grouping, Lancet **255**: 255, 1948.
11. Wannamaker, L. W., Denny, F. W., Rammelkamp, C. H., Jr., and Brink, W. R.: Use of Maxted's Method for Group Classification of Hemolytic Streptococci, Proc. Soc. Exper. Biol. & Med. **73**: 467, 1950.
12. Krumwiede, E., and Kuttner, A. G.: A Growth Inhibiting Substance for the Influenza Group of Organisms in the Blood of Various Species. The Use of the Blood of Various Animals as a Selective Medium for the Detection of Hemolytic Streptococci in Throat Cultures, J. Exper. Med. **67**: 429, 1938.
13. Rantz, L. A.: The Hemolytic Streptococci. Studies of the Carrier State in the San Francisco Area, With Notes on the Methods of Isolation and Serological Classification of These Organisms, J. Infect. Dis. **69**: 248, 1941.
14. Pike, R. M.: The Isolation of Hemolytic Streptococci from Throat Swabs. II. The Efficiency of Human and Rabbit Blood in Streaked and Streaked-Poured Plates, J. LAB. & CLIN. MED. **30**: 518, 1945.
15. Rammelkamp, C. H., Jr., and Dingle, J. H.: Pathogenic Streptococci, in Annual Review of Microbiology, Stanford, Cal., 1948, Annual Reviews, Inc.

A MECHANICAL SAMPLING DEVICE FOR USE WITH THE STANDARD ELECTROPHORESIS APPARATUS

DEMETRIOS A. RIGAS, PH.D., THOMAS J. MAUPIN, AND
CARL G. HELLER, M.D., PH.D.
PORTLAND, ORE.

A SIMPLE and inexpensive* mechanism has been devised for separating mechanically specific components from mixtures migrating in an electrophoretic field when only small amounts of material are available. Instruments for preparative purposes for dealing with large amounts of material have been described previously.[1-3]

Our apparatus can be used with any analytical electrophoretic cell and requires only that amount of material necessary for making the electrophoretic analysis. Thus it is possible to obtain the electrophoretic pattern and the separate fractions for analysis simultaneously. Separating and withdrawing the components after migration in the electrophoretic cell is followed and controlled by direct observation through the optical system of the electrophoretic apparatus. Construction of the device is simple and the cost of materials relatively low.

APPARATUS

Description.—The device consists of a long slender needle which can be inserted into the desired component without creating undue currents. The needle is attached to a syringe and the movement of the needle plus syringe is accomplished by a motor through which the speed of insertion may be controlled. The plunger of the syringe is connected with a second motor so geared that withdrawal of a component is accomplished without the production of convection currents.

The accompanying figure illustrates the device. A 5 ml. syringe (S), provided with a tripore adaptor (A), is attached to the needle. The syringe is mounted with the clamp (C_1) on a rigid support (B) capable of moving as a whole along the vertical frame (F). The supporting frame (F) is rigidly attached to the base (D), through which the apparatus can sit on the edge of the cold water bath with the tip of the syringe above the center of the electrophoretic cell. The movement of the syringe support is accomplished by the synchronous motor (M_1),† which drives a pinion moving a rack attached to (B). The motor is mounted on the supporting frame (F). The tip of the needle can thus be lowered to any desirable position in the cell, or withdrawn from it at a rate of 20 mm. per minute, a speed at which no disturbance of the crossed boundaries occurs. The plunger of the syringe is coupled to a threaded shaft through the special clamp (C_2) which allows the shaft to revolve without turning the plunger with it. The threaded shaft is driven by a keyed miter gear (G), which allows it to slide freely through

From the Division of Endocrinology of the Department of Medicine, University of Oregon Medical School.

Supported generously by grants from Schering Corporation, Bloomfield, New Jersey, and Ayerst, McKenna & Harrison, Limited, New Yok, New York.

Received for publication, Oct. 22, 1951.

*This apparatus was built at a total cost of $80.00, $25.00 of which was for the purchase of motors and materials used.

†"Telechron synchronous motor" Type B3 V 110/120 C60 MOD 59M31 R.P.M.1 W4. Manufactured by: Warren Telechron Co., Ashland, Mass. A second stator, taken from another similar motor, was added on each of the motors (M_1) and (M_2), to make possible the reversal of their rotation, at will.

by means of a keyway cut the full length of the shaft. This miter gear is driven by a synchronous motor (M_2) rigidly attached on the top of the supporting frame (F). The threaded shaft is provided with a half nut (E) attached on the syringe support (B), and which can be turned in or out of position at will. When in position, the motor (M_2) can pull the plunger to withdraw liquid from the cell, or push it to inject new protein solution in the bottom of the cell, if desired, at a speed slowly enough (0.1 mm. per minute, i.e., 0.1 ml. per minute with the 5 ml. syringe) to prevent turbulence. When (E) is out of position the plunger can be pushed down by hand to expel the withdrawn fraction into the receiving container, by turning the tripore adaptor (A). The apparatus can slide along the edge of the cold water bath, so that the needle can be inserted in either limb of the cell. The switchboard (H) is connected to the apparatus through a long cable, so that it can be carried at the far end of the schlieren camera, in which position it can be operated while the process is followed by direct observation on the screen.

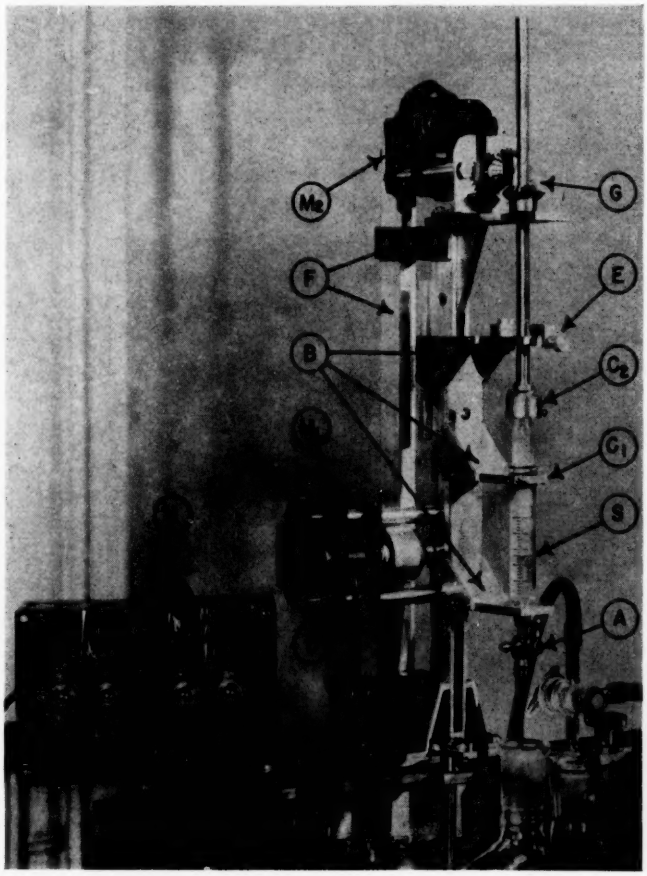


Fig. 1.—A photograph of the apparatus showing it in position ready for use.

RESULTS

Operation.—When the desirable migration and separation of the protein components in the cell have been achieved, the current is turned off, and the bottom cell shifted to the left to close the communication between the two limbs.

The limb to be fractionated is now opened, and the fractionator with the syringe raised to the upper limit is placed in position. The schlieren diaphragm is raised until the boundaries on each side of the fraction to be drawn from the cell have appeared on the screen and further scanned to a point near the base line. The upper border of the lower boundary (i.e., the lower border of the upper boundary, as they look on the reversed image on the screen) is now marked on the screen to be used as the border line beyond which the tip of the needle should not be allowed to pass. The syringe is lowered until the tip of the needle is halfway between the boundaries. When motor (M_2) is started and the fraction begins to be withdrawn, the upper boundary starts approaching the lower. The tip of the needle is kept halfway between the upper border of the lower boundary (previously marked on the screen) and the upper border of the upper boundary. Liquid is withdrawn until the boundaries merge. The tripore adaptor (A) is turned to the outlet position, and the half nut (E) is disconnected. The plunger is pushed down and the fraction is expelled. For best performance it is advisable to wet the syringe and the needle before starting the fractionation.

This technique can be successfully applied to a variety of problems. Enzymes, hormones, antibodies, etc., can be identified and separated from complex biological fluids and tissue extracts. In radioactive tracer studies, the specific activity found in fractions obtained by this method may yield important information on the metabolism of proteins, nucleic acids and similar compounds. The identification of a particular component in the electrophoretic pattern of the mixture where it may exist, makes possible the use of the electrophoresis as a guide in chemical purification. Important information was obtained by this method, on the nature of urinary proteins of normal subjects.[4]

SUMMARY

An apparatus to be used for obtaining samples from the standard electrophoretic cell, applicable in situations in which small quantities of material are available, has been described. The use of the equipment and possible applications of the method have been presented.

REFERENCES

1. Tiselius, A.: Electrophoretic Analysis and the Constitution of Native Fluids, The Harvey Lectures **35:** 37-70, 1939-1940.
2. Svensson, H.: Electrophoresis by the Moving Boundary Method. A Theoretical and Experimental Study, Arkiv. Kemi Mineral. Geol. **22A:** No. 10, 1946.
3. Cann, J. R., Kirkwood, J. G., Brown, R. A., and Plescia, O. J.: The Fractionation of Proteins by Electrophoresis-convection. An Improved Apparatus and Its Use in Fractionating Diphtheria Antitoxin, J. Am. Chem. Soc. **71:** 1603-1608, 1949.
4. Rigas, D. A., and Heller, C. G.: The Amount and Nature of Urinary Proteins in Normal Human Subjects, J. Clin. Investigation **30:** 853-861, 1951.

A COMPARISON OF SAPONIN AND AEROSOL AS HEMOLYTIC AGENTS IN THE DETERMINATION OF BLOOD GASES

STEVEN M. HORVATH, PH.D., AND DORIS W. KNAPP, M.S.
IOWA CITY, IOWA

YIENGST[1] has recently reported that a solution of 0.25 per cent aerosol (dioctyl sodium sulfosuccinate) can be substituted for the urea-albumin reagent[2] as a hemolytic agent for the manometric determination of the oxygen content of blood. However, since he did not have available a satisfactory saponin, Yiengst was unable to make a complete analysis of the effective substitution of aerosol for saponin. Furthermore, possible interference with carbon dioxide determinations in the combined oxygen-carbon dioxide method was not determined.

Blood was obtained from the right ventricle or femoral artery of dogs in quantities of 30 to 50 milliliters. Determinations of oxygen or oxygen-carbon dioxide were made by appropriate manometric methods.[3] Each sample was analyzed in duplicate using 0.25 per cent aerosol, one Merck saponin, and two Eastman Kodak saponins as the hemolytic agents. The Merck saponin was of prewar stock which has been used in this laboratory as a standard of comparison against presently available saponins. The duplicate analyses had a variation of less than 0.05 volume per cent oxygen on 1 ml. samples of blood. At least one complete comparison with all these agents was made by each of two operators. The results of the oxygen determinations are presented in Table I. The two lots of Eastman saponin provided as thorough a hemolytic

TABLE I. BLOOD OXYGEN VALUES* OBTAINED WITH VARIOUS HEMOLYTIC AGENTS

SAMPLE	NO. OF ANALYSES	AEROSOL 0.25%	SAPONIN NO. 1	SAPONIN NO. 2	SAPONIN NO. 3	SAPONIN MEAN
Combined method—Oxygen and Carbon Dioxide						
Arterial	12	16.34	16.26	16.18	16.23	16.23
Venous	9	12.33	12.22	12.37	12.15	12.24
Venous	9	12.45	12.47	12.42	12.45	12.45
Oxygen Alone						
Venous	4	14.44	14.47	14.37		14.42
Venous	5	13.98	13.50	13.49		13.50
Venous	6	10.51	10.54	10.48		10.52

No. 1.—Merck, prewar.
No. 2.—Eastman Kodak Practical.
No. 3.—Eastman Kodak Practical.
*All values are expressed in volumes per cent.

action as did the standard saponin. In general, all of the hemolytic agents appeared to be equally effective in both of the two methods for oxygen determination. There was a tendency for aerosol to give slightly higher values for oxygen in several instances. The mean difference was 0.12 volume per cent,

From the Department of Physiology, and the Cardiovascular Laboratory, College of Medicine, State University of Iowa.
Received for publication, Oct. 22, 1951.

standard deviation 0.10. A similar observation was made by Yiengst[1] in his comparison of aerosol and urea-albumin reagent. This discrepancy could not be explained. Aerosol did not interfere with the determination of carbon dioxide in blood (Table II).

TABLE II. CARBON DIOXIDE CONTENT OF BLOOD* (MM PER LITER) WHEN VARIOUS HEMOLYTIC AGENTS ARE EMPLOYED

SAMPLE	NO. OF ANALYSES	AEROSOL 0.25%	SAPONIN
Arterial	12	17.64	17.71
Venous	9	17.35	17.44
Venous	9	18.62	18.61

*Mean values.

It would appear that 0.25 per cent aerosol is a satisfactory substitute for saponin as a hemolytic agent. Its major disadvantages lie in the necessity of having an additional solution on hand for preparing each day's working reagent, and in the sporadic occurrence of high oxygen values.

The technical assistance of Mrs. Violet L. Tabler and Misses Margrieta Delle and Elizabeth Drennen is gratefully acknowledged.

REFERENCES

1. Yiengst, M. J.: A New Hemolytic Agent for the Manometric Determination of the Oxygen Content of Blood, Science **112**: 205, 1950.
2. Hiller, A., Plazin, J., and Van Slyke, D. D.: Substitutes for Saponin in the Determination of Oxygen and Carbon Monoxide of Blood, J. Biol. Chem. **176**: 1431-1437, 1948.
3. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Volume II, Methods, Baltimore, 1932, The Williams and Wilkins Company.

SPECTROPHOTOMETRIC ANALYSIS OF CARBOHYDRATES AND STUDY OF ANTHRONE REAGENT

R. E. Zipf, M.D., and A. L. Waldo, M.S.
Dayton, Ohio

THE reducing methods for the determination of the glucose content of biological fluids gives inordinately high results because there are substances other than glucose present that are reduced by the reagents. In blood, these non-carbohydrate reducing substances are chiefly glutathione, ergothione, uric acid, creatinine, creatine, and ascorbic acid. Most of the early data in American literature on blood sugar content were obtained by the Folin-Wu-copper-reduction method,[1] but it is gradually being superseded by more specific methods.[2, 3] The concentration of just glucose alone in normal blood under postabsorptive condition is 60 to 80 mg. per 100 ml. according to Folin and Wu[1] with their protein-free filtrate. The Folin method gives values from 90 to 110 mg. per 100 milliliters. The Somogyi and Kingsley methods[2, 3] give normal values of 70 to 100 mg. per 100 milliliters. The anthrone reagent of Dreywood[4] is specific for carbohydrates and, therefore, it has been adapted for the determination of glucose in blood, no matter which protein-free filtrate is employed. The normal values obtained by our modification of Dreywood's method are 65 to 90 mg. glucose per 100 ml. whole blood.

Spectrophotometric studies of the reaction of anthrone reagent with carbohydrates confirms that initially water is split from the carbohydrate with the formation of a hydroxyaldehyde which subsequently is converted to a furfural derivative.[5-8] Anthrone is the keto form of a tautomeric pair of which the enol form is anthranol. The anthranol form is highly reactive while the ketonic form, or anthrone, is indifferent to most reagents. In view of the classical Molisch test, which uses alpha-naphthol in concentrated sulfuric acid to test for carbohydrates, it seemed reasonable that anthranol might be the active form in this anthrone-carbohydrate reaction.

PROCEDURE

The following method has been found to yield consistent as well as precise results if the directions are followed implicitly.

Reagents.—

1. Sulfuric acid, N/12.
2. Sodium tungstate, 10 per cent, aqueous.
3. Anthrone reagent: To 25.0 ml. of distilled water add carefully 475.0 ml. of concentrated sulfuric acid. When this is cool, add with stirring 1.00 Gm. anthrone (Eastman, Organic Chemicals, Distillation Products Industries, Rochester, N. Y.). This should be prepared fresh every four to five days.

From the Biochemical Laboratory, Miami Valley Hospital.
Received for publication, Oct. 22, 1951.

4. Sulfuric acid, 1:1; for dilution, add to one part distilled water carefully one part sulfuric acid.

All pipettes, flasks, test tubes, and cuvettes must be scrupulously clean of lint, filter paper fibers, or any other carbohydrate material, since obviously they interfere with the analysis.

Analysis, Macro.—

1. Into a test tube or small flask pipette 8.0 ml. of N/12 sulfuric acid, add 1.0 ml. of whole oxalated blood.

2. Add 1.0 ml. of 10 per cent sodium tungstate, mix, let stand about ten minutes, then filter.

3. Into a 50-ml. Pyrex Erlenmeyer flask add 10.0 ml. of anthrone reagent, 1.0 ml. of protein-free filtrate, and immediately add 4.0 ml. of distilled water. Swirl to mix thoroughly.

4. Let the solution stand and cool for full color development at least fifteen minutes. If the tubes stand longer (forty-eight hours, stoppered) no further color change is noted.

5. Pour the mixture into a cuvette and read spectrophotometrically at 620 mμ or with a red filter. The reference liquid or blank must be the same solution that was used to make the standard curve; either distilled water or 10 ml. of reagent with 5 ml. of water.

6. When the concentration of sugar is higher than can be read, dilute 4.0 ml. of the green reaction mixture with 4.0 ml. of the 1:1 sulfuric acid-water mixture. Multiply the milligram per 100 ml. found by two.

Analysis, Micro.—

1. Into a 12-ml. centrifuge tube pipette 0.8 ml. N/12 sulfuric acid, 0.1 ml. oxalated whole blood, and 0.1 ml. 10 per cent sodium tungstate.

2. Mix, centrifuge ten to fifteen minutes at 2,500 r.p.m.

3. To 10.0 ml. of the anthrone reagent, which is in a 50-ml. Erlenmeyer flask, add 0.5 ml. of filtrate, and immediately 4.5 ml. of distilled water. Quickly swirl the contents to mix.

4. Let cool and read in the same manner as in the macroanalysis. Multiply the milligram per 100 ml. found by two.

Standard Curve.—

1. A stock solution of glucose is prepared by dissolving 1.0000 Gm. of chemically pure dry glucose in 500 ml. of distilled water in an appropriate volumetric flask.

2. When 12, 8, 5, 4, 3, and 0 ml. of this stock solution are diluted, respectively, to 100 ml., solutions are obtained which are equivalent to Folin-Wu or 1:10 protein-free filtrates of blood specimens containing 240, 160, 100, 80, 60, and 0 mg. glucose per 100 milliliters.

3. The above diluted solutions are treated the same as the blood filtrate, and carried through the preceding procedure.

4. The per cent transmission is plotted against the concentrations on semilogarithmic paper. The points should fall in a straight line. It is advisable to check the accuracy of these points with 4 or 5 other 1.0000 Gm. standard solutions.

EXPERIMENTAL AND DISCUSSION

The specificity of the reagent[4, 9] was again confirmed by us. The reagent gave a positive reaction with mono-, di-, and polysaccharides which can be converted to the original sugar in strong acid. No noncarbohydrate tested gave the green color reaction.

The reaction is dependent upon the heat developed when the reagent and water solutions are mixed.[8] Therefore, it was found that actually the size of the reaction tube is important. Less color is developed in a very small or a very large container due to rapid cooling and consequently incomplete reaction. We found a 50-ml. Pyrex Erlenmeyer flask most convenient and best for reproducible results. When the reaction is complete and the mixture cool, it is poured

into cuvettes for reading. We used the Model B, Beckman spectrophotometer for initial and experimental studies, and later routine runs on blood and cerebrospinal fluid glucose have been carried out with a 6A, Junior Coleman spectrophotometer and also a Cenco-Sheard-Sanford photelometer with good results.

The Folin-Wu filtrate made from cerebrospinal fluid gave a cloudiness to the final reaction mixture. We found that 4.0 ml. distilled water, 1.0 ml. cerebrospinal fluid, and 5.0 ml. freshly made 5 per cent trichloroacetic acid gave a protein-free filtrate which produced no cloudy reaction.

This anthrone method has been used in our diagnostic laboratories about a year with excellent results, and the method is much less time consuming[10, 11] than the previously used Somogyi method.[2]

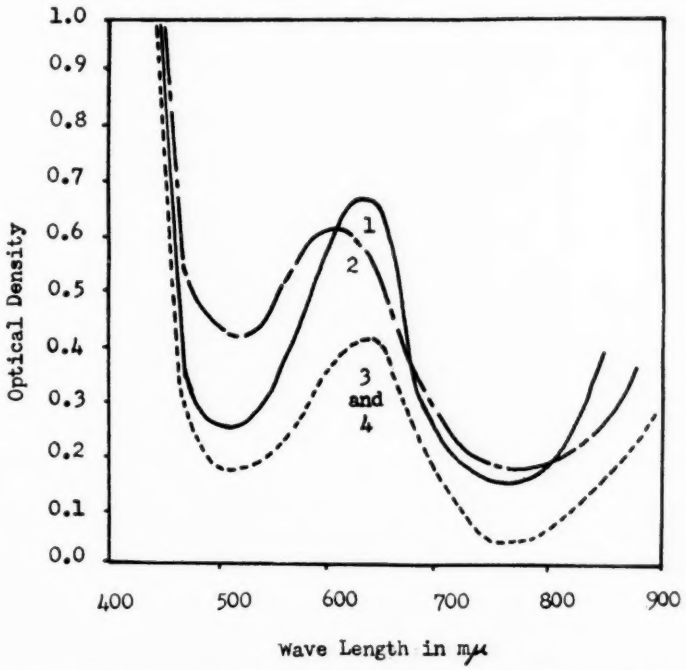


Fig. 1.—Comparison, of absorption spectral curves of the products resulting from the reaction of (1) gelatin, (2) furfural, (3) glucose with anthrone reagent, and (4) glucose with anthranol in sulfuric acid.

Our apparatus for dispensing the strongly acid reagent is a Cenco pressure-bulb type burette of 50-ml. capacity, with a 2-liter reservoir and all glass connections.

The following study of the mechanism of the reaction was undertaken in order to find whether the enol form, anthranol, was the reactive compound.

Anthranol was prepared in about 10 per cent yield by refluxing anthrone in 10 to 15 per cent aqueous sodium hydroxide for two to three hours, cooling to a low temperature with an ice-hydrochloric acid mixture and slowly, with stirring, acidifying with 1N sulfuric acid. The anthranol thus formed was quickly filtered and kept in an inert atmosphere of nitrogen.

The absorption spectral curves (Fig. 1) derived from the reaction of anthrone reagent with redistilled furfural gave a similar curve as an equimolar solution of glucose, except there was a slight shift in the maxima with furfural at 600 μ and glucose at 620 μ. Both curves showed absorption minima at 520 mμ and 760 μ. These results then confirm Sattler and Zerban's[6] finding that it is furfural or a furfural derivative from the respective carbohydrate which reacts with the reagent.

The reaction of anthranol in sulfuric acid gives a spectral curve similar to that of the reagent which has stood about four weeks (Fig. 2), and they are dissimilar to the curve of freshly prepared anthrone reagent. This shows that anthrone slowly shifts to the enol form which is so sensitive to oxidation, that

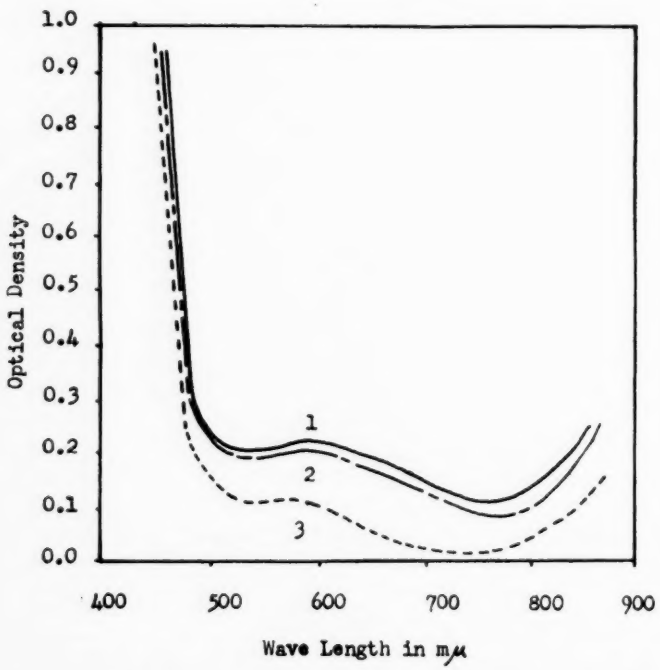


Fig. 2.—Comparison of absorption spectral curves of the products resulting from (1) anthranol in sulfuric acid, (2) 4-week-old anthrone reagent, (3) fresh anthrone reagent.

it may have suffered much decomposition, which accounts for the dark brown to purplish color in the long-standing reagent. The reaction of glucose with anthranol in sulfuric acid (Fig. 1) gives the same shape curve as with anthrone and sulfuric acid, showing the equilibrium that exists between the two forms. Thus the highly reactive enol form probably condenses with the carbohydrate-furfural derivative to produce the characteristic green color in dilute solutions and a deep blue color in more concentrated solutions.

It is interesting to note that the spectral curves of glucose, galactose, glutamic acid, gelatin, and the Folin-Wu filtrate all show absorption maxima and minima at the same wave length (Figs. 1 and 3). The curves derived from

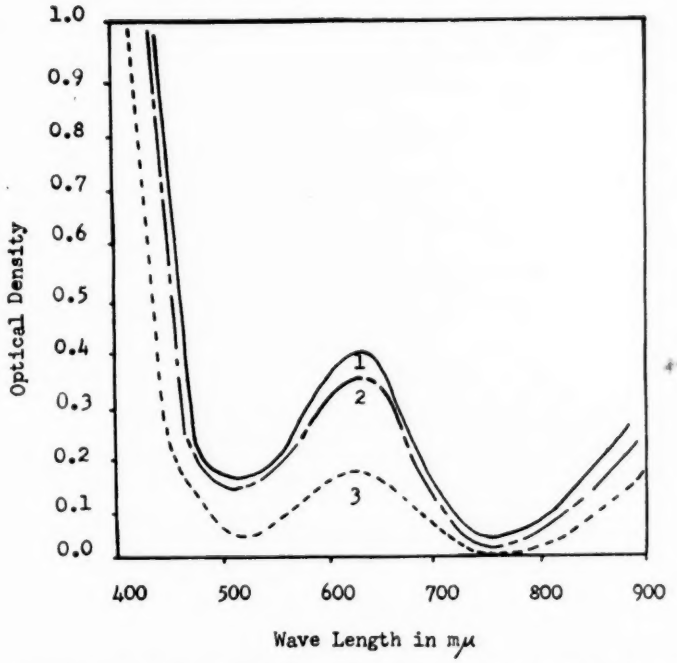


Fig. 3.—Comparison of absorption spectral curves of the products resulting from the reaction of (1) galactose, (2) the Folin-Wu filtrate, and (3) glutamic acid with anthrone reagent.

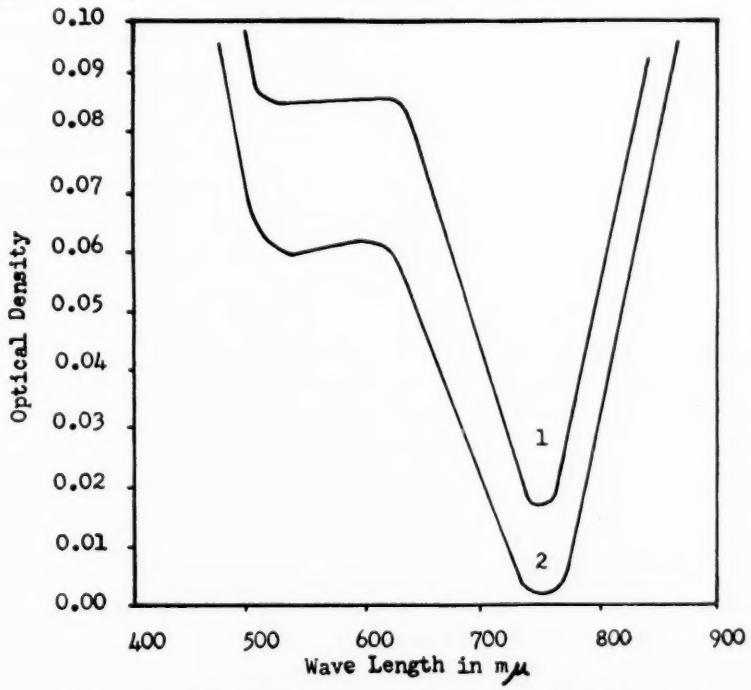


Fig. 4.—Comparison of absorption spectral curves of the products resulting from the reaction of (1) casein and (2) l-cystine with anthrone reagent.

casein and l-cystine appear identical (Fig. 4). Our spectral curves derived from the reaction of glucose and galactose with the anthrone reagent are identical with those found by Graff and co-workers.[12]

SUMMARY

An accurate macro- and microspectrophotometric analysis of glucose or carbohydrates in body fluids or other solutions is described with the use of the anthrone reagent.

The mechanism of the color development was determined. The carbohydrate-furfural derivative condenses with the highly reactive enol tautomer, anthranol.

REFERENCES

1. Folin, O., and Wu, H.: A System of Blood Analysis, J. Biol. Chem. **38**: 81, 1919.
2. Somogyi, M. J.: Determination of Blood Sugar, J. Biol. Chem. **160**: 69, 1945.
3. Kingsley, G. R., and Reinhold, J. G.: The Determination of True Glucose in Blood by Reduction of Ferricyanide, J. LAB. & CLIN. MED. **34**: 713, 1949.
4. Dreywood, R.: Qualitative Test for Carbohydrate Material, Ind. Eng. Chem. (Anal. Ed.) **18**: 499, 1946.
5. Sattler, L., and Zerban, F. W.: The Dreywood Anthrone Reaction as Affected by Carbohydrate Structure, Science **108**: 207, 1948.
6. Sattler, L., and Zerban, F. W.: Limitations of the Anthrone Test for Carbohydrates, J. Am. Chem. Soc. **72**: 3814, 1950.
7. Hurd, C. D., and Isenhour, L. L.: Pentose Reaction: I. Furfural Formation, J. Am. Chem. Soc. **54**: 317, 1932.
8. Wolfrom, M. L., Schultz, R. D., and Cavalieri, L. F.: Chemical Interactions of Amino Compounds and Sugars. III. The Conversion of d-Glucose to 5-(Hydroxymethyl)-2-furaldehyde, J. Am. Chem. Soc. **70**: 514, 1948.
9. Morris, D. L.: Quantitative Determination of Carbohydrates with Dreywood's Anthrone Reagent, Science **107**: 254, 1948.
10. Durham, F. W., Bloom, W. L., Lewis, G. T., and Mandel, E. E.: Rapid Measurement of Carbohydrate in Blood, Pub. Health Rep. **65**: 670, 1950.
11. Fetz, R. H., and Petrie, L. M.: The Anthrone Blood Sugar Method Adapted to Diabetes Case Finding in Multiple Screening Program, Pub. Health Rep. **65**: 1709, 1950.
12. Graff, M. M., Greenspan, E. M., Lehman, I. R., and Holechek, J. J.: Estimation of Nonglucosamine Polysaccharides of the Serum Proteins with the Anthrone Reagent, J. LAB. & CLIN. MED. **37**: 736, 1951.

MUCIN SOLUTION FOR ENHANCING THE VIRULENCE OF S. TYPHI

LUCY MISHULOW, B.A.
NEW YORK, N. Y.
ASSISTED BY LESLIE S. SHARPE, B.A., AND LILLIAN L. COHEN, B.A.

OUR experience with mucin solutions for the enhancement of the virulence of *S. typhi* is similar in many respects to that of Siler, Dunham, Longfellow, and Luippold.* We also found that different preparations of mucin vary considerably in their virulence-enhancing power; that autoclave-sterilization at more than 10 pounds pressure considerably reduced their potency; that sterilization at 100° C. by flowing steam for thirty minutes on three consecutive days gave a uniformly satisfactory product.

But in addition to that we found that mucin solutions sterilized at an acid reaction (pH 5.0) retain their potency on storage at 5° to 10° C. for a much longer period than those that are adjusted to pH 6.8-7.0 before sterilization, and that the addition of sterile 1 per cent glucose to the finished product just prior to the test contributes to the virulence-enhancing power of the mucin, possibly by increasing the rapidity of multiplication of the organisms.

METHOD

The method that we employed in the preparation of mucin solutions during the past ten years with uniformly good results is as follows:

To prepare one liter of 5 per cent mucin solution add 50 Gm. of mucin to 950 ml. of hot distilled water in a flask. Mix well. Put a paper cap on the flask and place the flask in a pot of boiling water for thirty minutes. Twirl flask every five minutes until mucin is dissolved. If any clumps of mucin do not dissolve within thirty minutes, grind them in a mortar and add to the bulk.

Then filter the solution through four thicknesses of fine cheesecloth (Bellevue Roll). Distribute the mucin solution into flasks making a shallow layer for quick sterilization. Sterilize in the Arnold, *without adjusting the reaction,* for thirty minutes on three consecutive days. Test for sterility. Stopper the flasks with sterile solid-rubber stoppers and store at 5° to 10° C. This preparation will remain potent for several months.

Before the test, warm the mucin solution until it flows freely. Adjust the reaction to pH 7.2-7.4 with normal NaOH. Add 50 per cent sterile dextrose solution to make 1 per cent of the carbohydrate, then place in boiling water and twirl for five minutes. Filter through monel metal wire-gauze 60 gauge. This will remove any small clumps that might clog the needles.

From Bureau of Laboratories, Department of Health, New York City.
Received for publication, Nov. 5, 1951.
*Immunization to Typhoid Fever, Am. J. Hygiene, Monograph Series No. 17.

The 5 per cent mucin solution may be used as is, or it may be diluted with Ringer's solution to 4 per cent by combining four parts of the 5 per cent mucin with one part of Ringer's solution.

In order to prevent the dilution of the mucin in the final preparation of the challenge culture, serial preliminary dilutions of the culture are made in Ringer's solutions, but the two dilutions before the final challenge dose and all subsequent dilutions are made in the mucin solution. For example, if the challenge dose is in 10^{-7}, then dilutions 10^{-5}, 10^{-6} and all subsequent dilutions are made in the mucin solution.

Using this method and the strains 42-A-58 or No. 779 of *S. typhosa*, the LD50 seldom exceeded twenty bacilli.

Erratum

In the January, 1952, issue of the JOURNAL, in the article entitled "Blood Pressure in the Aorta During Resection, and Repair of Coarctation of the Aorta" by Josiah Fuller, M.D., Bowen E. Taylor, M.D., O. Theron Clagett, M.D., and Earl H. Wood, M.D., Ph.D., the illustrations on pages 18 and 19 should be transposed. The illustration labelled Fig. 5 should be Fig. 4, and the one labelled Fig. 4 should be Fig. 5.